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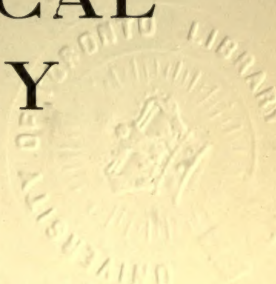
**PRACTICAL
PHYSIOLOGICAL CHEMISTRY**



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1921

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PRACTICAL PHYSIOLOGICAL CHEMISTRY



BY

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THIRD EDITION

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PREFACE TO THE FIRST EDITION

THIS book is intended to serve as a Guide to Junior Students attending a Class of Practical Physiological Chemistry, of approximately three months' duration. There is no subdivision into daily exercises, as the time available during the day and week varies in different schools. The teacher may therefore omit certain parts and supplement others, as he may deem advisable, and, to facilitate this, the book has been interleaved. All matter which does not bear directly upon the practical work has been excluded, only that amount of theoretical explanation being given which has seemed to us essential for the proper understanding of test or process respectively. A few literature references are given in the text, but these refer only to suitable books for reference in cases where a more complete description might be desired.

We have made use of such laboratory handbooks as were at our disposal, especially those of Huppert, Hoppe-Seyler—Thierfelder, Salkowski, and also of the various physiological journals and systematic text-books, especially the chemical articles in the "Text-book of Physiology," edited by E. A. Schäfer. Illustrations are given of some forms of apparatus mainly employed in physiological chemical work.

We have to thank Mr. Richard Muir for the sketches of apparatus and urinary crystals, and the Lithographers for the care taken in the reproduction of the spectra.

PREFACE TO THE THIRD EDITION

IN the present edition, the scope of the book has been extended so as to meet the requirements of senior and junior students of medicine and biology. In addition to providing material for the immediate needs of junior and senior classes, the writers hope that the book will prove serviceable to medical students in their later work. The more advanced portions of the volume have been printed in smaller type in

order to prevent undue expansion, or alternatively placed within brackets.

As in previous editions, practical instructions have been printed in italics, and theoretical statements in Roman type. The latter take the form of brief introductions to the majority of the chapters in the qualitative section. Students are advised to read these introductory sketches before carrying out the corresponding practical work. The writers have included this small amount of theoretical material with the object of aiding students in correlating information gained from lectures or reading with that acquired experimentally. To further this aim, references to readily accessible textbooks and other literature have been freely inserted. It is hoped that these references may also prove useful for students desiring more complete information on certain topics.

The rapid progress of physiological chemistry, and the ever increasing accumulation of methods render the task of selecting material a difficult one, and the selection given in this book is probably in many respects open to criticism. The writers regret that the subjects of gas analysis as applied to physiology and of dynamical as distinguished from descriptive physiological chemistry have received inadequate treatment. A satisfactory account of these subjects would have entailed an undue increase in the size and cost of the book.

While both writers are responsible for the work as a whole, all the new material in this edition, with the exception of pages 264 to 283 and 388, 389 (contributed by T. H. Milroy), has been written by J. A. Milroy.

We are deeply indebted to Dr. J. S. Jowett Lee for figures 1, 2, 7 to 9, and 12 to 20; to H. E. Miller for figures 5 and 6; and to J. Wylie, B.A., for figures 3 and 4. We beg to express our thanks also to Dr. Mansfield Clark for his permission to make use, in modified form, of his figure of the calomel-hydrogen electrode chain.

August, 1921.

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SECTION I
QUALITATIVE



PHYSIOLOGICAL CHEMISTRY

CHAPTER I

DETECTION OF THE ELEMENTS IN AN ORGANIC SUBSTANCE

THE substance must be freed from moisture, either, in the first place, by driving off the larger part in a drying oven, and then removing the last traces in a vacuum exsiccator, or, if there should be a tendency to decomposition, by drying in the exsiccator alone.

The elements of importance are carbon, hydrogen, nitrogen, sulphur, phosphorus, and the halogens; and their detection is carried out in the following ways:—

Preliminary Examination.

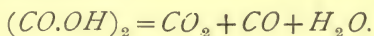
Many organic substances char on heating. Heat a small quantity of cane sugar in a dry test-tube. The substance melts, chars, and yields vapours having an odour more or less characteristic of burnt carbohydrate. Droplets of water will also be seen to condense on the walls of the test-tube. Again heat a particle of the sugar on platinum foil, or the lid of a quartz crucible. The substance first chars, and then undergoes complete combustion, leaving no residue, if the sugar be pure.

Carry out the same experiments with oxalic acid. This substance on being heated melts in its water of crystallis-

ation. When the latter is driven off, the white residue on further heating undergoes complete combustion without charring.

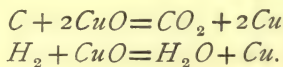
Some organic substances may be combined or mixed with inorganic material. When heated on platinum foil, these leave a residue, if the inorganic material be non-volatile. The nature of the residue may be determined by the methods of inorganic analysis. Organic compounds containing volatile metals, e.g. mercury, arsenic, or zinc, may leave no residue on incineration. In such cases other methods must be used for the destruction of the organic material. The presence of alkalis in the ash of an organic substance, such as sodium citrate, may be overlooked, since the fused alkalis form a transparent glassy layer on the surface of the platinum. Their presence may be readily detected by means of moistened red litmus paper applied to the cooled surface of the platinum.

Many organic substances also blacken owing to the separation of carbonaceous material, when heated with concentrated sulphuric acid. Carry out this test with small quantities of starch or cane sugar, and of oxalic acid. Note in the latter case the absence of distinct charring and the evolution of gas consisting of carbon dioxide, and carbon monoxide.



A more complete description of the effects of heat on certain organic substances of physiological importance will be found on pages 413 and 414.

The foregoing methods of detecting the presence of carbon are limited in their application. The most certain method is based on the oxidation of carbon to carbon dioxide, and of hydrogen to water by means of cupric oxide.



The method is carried out in the following way:—

[I. CARBON AND HYDROGEN.]

Powder a few crystals of cane sugar in a dry mortar, add about ten times as much finely powdered water-free cupric oxide (previously heated and preserved in exsiccator), and mix thoroughly. Quickly place the mixture in an absolutely dry test-tube which has a rubber stopper with a glass tube passing just through it, and on the other side leading into a small bottle containing clear baryta water. Now, heat tube over gas flame, and as the substance undergoes oxidation, water drops are seen to condense in the colder part of the tube, and barium carbonate begins to separate out in the hydrate solution.

II. NITROGEN, SULPHUR, AND THE
HALOGENS.

When an organic substance is heated with sodium, nitrogen, if present, is converted into sodium cyanide (together with some thiocyanate, if sulphur be also present, and the quantity of sodium be insufficient), sulphur mainly or entirely into sodium sulphide, and the halogens into sodium chloride, bromide or iodide. These salts may be identified by the usual reactions in the filtered aqueous solution of the cooled product of incineration.

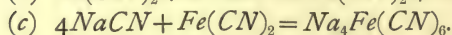
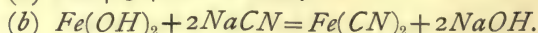
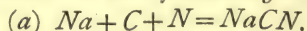
The method is carried out in the following way:— Drop a few particles of the organic substance (about 0.02 g.) into a hard glass test-tube held vertically, taking care that none of the substance adheres to the sides of the test-tube. Then add a piece of clean, dry sodium of about 4 mm. diameter. Heat at first gently until the reaction slackens, then more strongly until the bottom of the test-tube becomes red. Allow the test-tube to cool completely, then add a small quantity of ethyl alcohol to get rid of the excess of sodium; after some minutes dilute with about 10 c.c. of water, heat to the boiling point, and filter. When

diluting with water care should be taken in case some free sodium still remains unconverted into sodium ethylate.

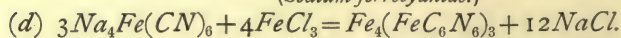
The filtrate is then tested for

(1) **Nitrogen** in the form of sodium cyanide (Lassaigne's method). Two or three drops of a solution containing 0.5 per cent. ferrous sulphate, and 0.5 per cent. ferric sulphate or chloride are added to a small portion of the filtrate. A little caustic soda may be added if the solution is not sufficiently alkaline; but this addition is usually unnecessary. The alkaline suspension is then boiled, cooled under the water tap, and acidified slightly with pure hydrochloric acid. A blue solution containing particles of Prussian blue in suspension is obtained if nitrogen were present in the original substance. If only traces of cyanide be present, a green solution is obtained from which particles of Prussian blue separate out on standing. If the solution remains pure yellow and no Prussian blue separates on standing, the organic substance probably contains no nitrogen. The presence of sulphur interferes with, and may even prevent, a positive reaction, owing to the formation of thiocyanate. In the latter case, another small portion of the filtrate should be faintly acidified with hydrochloric acid, and tested with dilute ferric chloride. If thiocyanate be present, a deep red colour is obtained. This result indicates the presence of both nitrogen and sulphur. In this event, the presence of nitrogen may be confirmed by repeating the incineration, using twice as much sodium as in the first experiment, and then preparing Prussian blue by the method already described.

The reactions involved in Lassaigne's test may be summarised in the following equations:—



(Sodium ferrocyanide.)



(Ferric ferrocyanide
or Prussian blue.)

(1^a) THE SODA LIME TEST.

The soda lime test may also be used for the detection of nitrogen, although it is not quite so reliable as Lassaigne's method.

Mix up a small quantity of the organic substance thoroughly, with at least twenty times as much soda lime, and heat strongly over flame. Test vapours coming off for ammonia by holding over the mouth of the tube a piece of moistened red litmus or turmeric paper, or a rod dipped in HCl. The strong ammoniacal odour is also distinct.

(2) Sulphur.

(a) A few drops of a freshly prepared solution of sodium nitro-prusside are added to another portion of the filtrate. The appearance of a reddish-violet colour indicates the presence of sulphide. If sulphide be absent, the fluid becomes yellow.

(b) Add a drop of lead acetate solution to a little caustic soda solution, heat until the precipitate of lead hydrate has dissolved, and add the resulting clear solution to another fraction of the filtrate. The formation of a black precipitate of lead sulphide indicates the presence of sodium sulphide.

(3) The Halogens.

(a) CHLORINE. — A little silver nitrate solution is added to another portion of the filtrate, previously acidified with nitric acid. A white precipitate readily soluble in ammonia may consist of silver chloride, cyanide or thiocyanate. A light yellow precipitate soluble only in large excess of ammonia may consist of silver bromide or iodide. If the substance has already been found to be free from nitrogen, the presence of cyanide is excluded. On the other hand, if the substance contain nitrogen, a fresh portion of the filtrate should be strongly acidified with nitric acid and boiled for about two minutes in order

to get rid of cyanides and sulphides before adding the silver nitrate. If bromides or iodides be present, some bromine or iodine is evolved during the heating with nitric acid.

(b) BROMINE and (c) IODINE.—Slightly acidify another portion of the filtrate with hydrochloric acid, add one or two drops of chlorine water and shake up with chloroform or carbon disulphide. If bromine alone is present, the chloroform or carbon disulphide dissolves it, yielding a yellow, orange, or reddish-brown solution according to the amount of bromine present. If iodine alone is present, its solution in chloroform or carbon disulphide has a violet colour. If both halogens be present, this method yields a doubtful result. Care must be taken to avoid the addition of excess of chlorine, which unites with bromine or iodine to form colourless compounds.

The presence of iodide may also be detected by the following tests:—Acidify a small portion of the filtrate with dilute sulphuric acid, add one to three drops of 0.2 per cent. sodium nitrite, and divide the solution into two portions. Shake up one of these with chloroform. The chloroform dissolves the free iodine, forming a violet coloured solution. Add a small quantity of starch mucilage to the other portion, a blue colour results due to the formation of iodide of starch.

III. PHOSPHORUS.¹

(a) Take about 0.2 gm. of the nucleo-protein of the thymus gland (free from inorganic phosphates), or a larger quantity of dried caseinogen, and mix up thoroughly in a mortar with about twenty times its quantity of a soda-salt-petre mixture (1 Na_2CO_3 to 2 KNO_3).²—Incinerate

¹ A preliminary incineration should be carried out by the demonstrator, and the aqueous extract given out to the class.

² In this, as in all cases, the reagents employed must be free from any trace of the element, for which examination is being made.

this in a porcelain crucible gradually, until the mass has become white. Cool, dissolve residue in as small a quantity of hot water as possible, and pour solution into a porcelain basin; add pure HCl to drive off CO_2 ; filter. Divide filtrate into two portions. Make one portion alkaline with ammonia, and precipitate the phosphate with magnesia mixture as ammonium-magnesium phosphate, which separates out in a white, more or less crystalline form. The alkaline phosphates present in the hot water extract may be detected also in another way. The other portion of the filtrate, on being made acid with HNO_3 and heated, will give a yellow precipitate of ammonium phospho-molybdate when a fairly large quantity of ammonium molybdate is added to the warm solution. It is advisable in this case to add the solution suspected of containing phosphate to the ammonium molybdate, as the precipitate of ammonium phospho-molybdate is soluble in excess of dissolved phosphate.

The foregoing method may also be used for the detection of sulphur. Any sulphur present in an organic substance is converted into sulphate by fusion with soda-saltpetre mixture. The sulphate present in the acid filtrate, obtained as already described, is then precipitated as barium sulphate by the addition of barium chloride.

(b) The presence of phosphorus may be more conveniently demonstrated by Neumann's method, which consists in oxidising the organic material by heating the substance with a mixture of equal parts of concentrated sulphuric and nitric acids (see p. 253).

IV. IODINE.

Powder two or three thyroid tabloids in a mortar and transfer to nickel or porcelain crucible. Moisten with a little distilled water and add about twice the quantity of pure NaOH (from metallic sodium). Heat gradually until the mass is completely charred, then add at short

intervals about an equal quantity of potassium nitrate. Continue heating until mass just becomes white, then allow to cool, dissolve in hot water, filter into test-tube, cool, acidify with H_2SO_4 , and add an equal quantity of chloroform. On shaking the mixture, the iodine is extracted by the $CHCl_3$ and quickly settles down as a rose-red layer at the bottom of the test-tube.

From this preliminary series of tests for the elements, one is in a position to surmise at least the nature of the substance being examined. Thus a substance found to be free from N will also usually be free from sulphur, phosphorus, and iodine, and will probably either be a carbohydrate or fat, or some nitrogen-free derivative of protein. Methods for identifying these will be described in subsequent chapters. If the body contain N, and give a very distinct phosphorus reaction, it probably will be of the nature of a phospho-protein or lecithin. If it give a distinct iodine reaction, then it is probably a thyroid preparation of some kind. The detection of sulphur simply shows that the body is a protein or a derivative. If it be large in amount, then one suspects the presence of a body of the keratin group (to a lesser degree, also, serum albumin), taurine, or cystine; if only a mere trace—peptone. Of course, such a preliminary investigation can never be decisive. It only points out a possible fruitful line of examination. The foregoing inferences are only justifiable if the investigation be limited to organic substances of physiological importance.

The following organic substances may be used for testing students in the application of the methods just described:—Starch, cane sugar, sodium potassium tartrate (for detection of C.H.O. and ash), uric acid, amino-acids, urea, etc. (C.H.N. and O.), salicyl sulphonic acid (C.H.S. and O.), protein, cystine, thiourea, or sulphanilic acid (C.H.S.N.), phospho-proteins, such as caseinogen, nucleoproteins, sodium nucleate (C.H.N.P. and S. in some of these substances), chloral, eosin ($C_{20}H_8O_5Br_4$), erythrosin ($C_{20}H_8O_5I_4$), and thyroid substance (the last four substances for the halogens).

CHAPTER II

CARBOHYDRATES AND ALLIED SUBSTANCES

THESE, on being examined in the way described in the previous chapter, are found to contain carbon and hydrogen; but are free from nitrogen, sulphur, phosphorus, and iodine.

INTRODUCTION

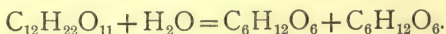
The simplest carbohydrates, known as monoses or monosaccharides, may be chemically defined as aldehydes or ketones of polyhydric alcohols. With the exception of some methyl derivatives, they all have the empirical formula $(\text{CH}_2\text{O})_n$. n may have any value from 2 to 9. The most important members of the group, in which n has the value 5 or 6, are known as pentoses (aldo- and keto-pentoses) and hexoses (aldo- and keto-hexoses) respectively.

One or more asymmetric carbon atoms are present in all monoses, consisting of an open chain of three or more carbon atoms, and consequently these sugars should be capable of existing in several optically active stereo-isomeric forms. The number of stereo-isomerides of the aldoses may be calculated from van 't Hoff's formula 2^n , in which n represents the number of asymmetric carbon atoms. Thus the aldo-pentoses contain 3, the aldo-hexoses 4 asymmetric carbon atoms, and therefore 2^3 or 8 optically active aldo-pentoses, and 2^4 or 16 optically active aldo-hexoses are possible. The 2^n stereo-isomerides form half as many optically active pairs, each pair consisting of two sugars of equal, but opposite, rotatory power. These are open chain forms. The number of

possible stereo-isomerides is greatly increased when closed chain forms of the aldo-hexoses are included. The number of hexoses found in nature is relatively small—namely, three aldo-hexoses—glucose, galactose, and mannose; and two keto-hexoses—fructose or levulose¹ and sorbose. The pentoses are not generally found in the free state except in urine; but either in complex combination, as in certain nucleic acids or as polysaccharides of high molecular weight known as pentosans. The most important pentoses are xylose, arabinose, and ribose.

Aqueous solutions of the majority of the sugars—pentoses and hexoses—show mutarotation, *i.e.* the optical rotatory power of their freshly prepared solutions gradually changes, usually diminishing, but sometimes increasing, until a constant value is reached. The magnitude and sign of the final specific optical rotation is characteristic for each sugar, and is constant within certain limits of concentration, and for a stated temperature.

When two hexoses undergo condensation with the elimination of the elements of one molecule of water, disaccharides or hexo-bioses, containing twelve carbon atoms, and having the general formula ($C_{12}H_{22}O_{11}$) are formed. Conversely, when disaccharides are hydrolysed, either by enzymes or by dilute acids, they break down into two hexoses.



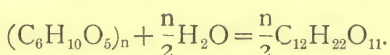
The following table contains the chief disaccharides, with the products of their hydrolysis. Maltose and lactose possess reducing properties, since they contain a free aldehyde group; while cane sugar has no reducing properties, since it contains no free aldehyde group:—

Disaccharides.	Products of Hydrolysis.
Maltose	Glucose and Glucose.
Lactose	Glucose and Galactose.
Cane Sugar (Sucrose) .	Glucose and Fructose or Levulose. ¹

¹ Also written lævulose.

Tri- and tetra-saccharides are also known, but are not of great physiological importance. The mono- and di-saccharides resemble one another in being crystalline, readily soluble in water, and sweet. They therefore form one natural class known as sugars.

The polysaccharides are amorphous, tasteless substances, either insoluble in water or much less soluble than the mono- and di-saccharides, and of high but unknown molecular weight, having the general formula $(C_6H_{10}O_5)_n$ in the case of those derived from hexoses. Similar derivatives of the pentoses known as pentosans are present in many gums. On complete hydrolysis with acids they yield mono-saccharides. Many of them are also converted into disaccharides by the action of enzymes.



The chief polysaccharides and the products of their complete hydrolysis by acids are contained in the following table :—

Polysaccharides.	Final Products of Hydrolysis.
Starch	Glucose.
Glycogen	Glucose.
Dextrins	Glucose.
Inulin	Fructose or Levulose.
Cellulose	Glucose.
Gums { Pentosans	Pentoses, } in varying proportions.
{ Galactosans	
Mannosans	Mannose accompanied by other hexoses.

On reduction, the hexoses yield hexa-hydric alcohols ; on oxidation, the aldo-hexoses yield successively mono- and di-basic acids, containing the same number of carbon atoms

as the original sugar ; while the keto-hexoses are split at the ketonic group into two hydroxy-acids.

The following table contains the chief hexoses and the products of their reduction and oxidation :—

Hexahydric Alcohol.	Hexose.	Monobasic Acids.	Dibasic Acids.
$\text{CH}_2\text{OH}(\text{CHOH})_4\text{CH}_2\text{OH}.$	$\text{CH}_2\text{OH}(\text{CHOH})_4\text{CHO}.$	$\text{CH}_2\text{OH}(\text{CHOH})_4\text{CO.OH}.$	$\text{COOH}.\text{(CHOH)}_4\text{CO.OH}.$
Sorbitol Dulcitol Mannitol	Glucose Galactose Mannose	Gluconic acid Galactonic acid Mannonic acid	Saccharic acid Mucic acid Manno-saccharic acid
Sorbitol and Mannitol	Fructose $\left\{ \begin{array}{l} \text{CH}_2\text{OH}(\text{CHOH})_3 \\ \text{CO.CH}_2\text{OH} \end{array} \right.$	Glycollic ($\text{CH}_2\text{OH.CO.OH}$) and Trihydroxybutyric [$\text{CH}_2\text{OH}(\text{CHOH})_2\text{CO.OH}$] acids	

Glucuronic (Glycuronic) acid [$\text{CO.OH}.\text{(CHOH)}_4\text{CHO}$] forms an intermediate stage in the oxidation of gluconic to saccharic acid. It is of great physiological interest, being frequently present in the urine as paired compounds with a large number of substances (see pages 239-241). It may be prepared by the reduction of saccharic acid ; but the yield obtained by this method is so small that the acid is best obtained from one or other of its paired compounds.

Amino - hexoses [$\text{CH}_2\text{OH}.\text{(CHOH)}_3\text{CH.NH}_2\text{CHO}$] are found amongst the products of acid hydrolysis of the mucins or glucoproteins, and a number of other bodies of high molecular weight, such as chitins, chondrin, and certain mucoids.

A. MONOSACCHARIDES ($\text{C}_n\text{H}_{2n}\text{O}_n$).

The most important one is the hexose, glucose. Most of the reactions depend upon the fact that it possesses an aldehyde group. Another important hexose is fructose or levulose, which contains a ketone group.

I. HEXOSES ($C_6H_{12}O_6$).

1. **Glucose (grape sugar or dextrose)** is the aldehyde of the hexatomic alcohol, sorbitol.

Many of the most commonly employed tests depend upon the power which glucose possesses of reducing metallic oxides in hot alkaline solution.

EFFECT OF HEAT ON DRY GLUCOSE.

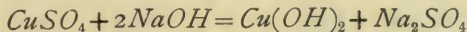
Heat gradually a small quantity of anhydrous glucose in a dry test-tube. The sugar melts to form a clear, colourless fluid which rapidly becomes brown on further heating, evolving water and a number of volatile and gaseous products having the characteristic odour of burnt sugar.

Glucose (1 per cent. solution).

(a) TROMMER'S TEST.

When carrying out Trommer's test, it is advisable to do a control experiment with the reagents.

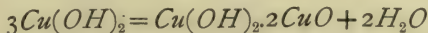
Place in one test-tube about 5 c.c. of water, in another the same volume of the solution of glucose. Add to the fluids in each test-tube about half their volume of caustic soda solution, and drop by drop a solution of copper sulphate as long as the precipitate of cupric hydrate readily dissolves in the glucose solution (about 20 drops to each). It will be noted that in the control test the cupric hydrate precipitate remains undissolved.



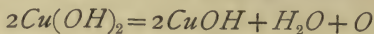
(Soluble in glucose solution).

Heat both solutions gradually to the boiling-point. A brownish-black precipitate separates out from the former alkaline suspension; while a yellow precipitate (cuprous hydrate) which soon becomes red (cuprous oxide) separates out from the latter solution.

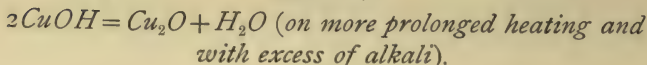
(1) Control with reagents.



(Black hydrated cupric oxide).

(2) *Reduction in presence of glucose.*

(Yellow).



(Red).

Part of the cuprous oxide also appears to be reduced to copper, especially if the solution of glucose be concentrated (Monnet).

It is of great importance to have little or no cupric hydrate undissolved, otherwise, on heating, the colour of the precipitated cuprous salts is obscured by the formation of black cupric oxide.

EXPLANATORY NOTES.

(1) Polyhydric alcohols, e.g. glycerol and mannitol dissolve cupric hydrate; but no reduction occurs on heating. This result indicates that the reducing power of glucose is due to the fact that it is an aldehyde.¹

Place about 5 c.c. of water in a test-tube. Add about half this volume of caustic soda, 20 drops of copper sulphate solution and a few drops of glycerol, or of an aqueous solution of mannitol. Note that the precipitate of cupric hydrate dissolves in the cold to form a clear blue solution, which remains unchanged on heating, if the glycerol or mannitol be pure.

Cupric hydrate also dissolves in the presence of neutral salts of many hydroxy-acids, such as tartaric acid, and the acids (e.g. saccharic) resulting from partial oxidation of the hexoses. These solutions remain unchanged on heating.

Repeat the foregoing experiment, adding sodium potassium tartrate in place of glycerol.

(2) If the caustic soda used in Trommer's test be replaced by ammonia, the cuprous oxide remains in solution.

Place about 5 c.c. of the glucose solution in a test-tube, add an equal volume of 1 in 4 ammonia and about 20 drops of copper sulphate solution, and boil for a few minutes. The solution is gradually decolourised, and finally becomes light yellow.

Ammonium salts, therefore, interfere with the separation of cuprous oxide in Trommer's and Fehling's tests.

(b) FEHLING'S TEST.

In order to overcome the disadvantage of excess of undissolved cupric hydrate, just referred to, an alkaline solution of

¹ All aldehydes, which are sufficiently soluble in water (e.g. formaldehyde and acetaldehyde) give Trommer's and most of the other reduction tests. Formates and chloroform, which yields formate when heated with alkali, also reduce cupric hydrate.

sodium potassium tartrate is added to the cupric sulphate solution, and this constitutes what is termed Fehling's solution, which is free from precipitate and is of a deep blue colour.

The reagent should be freshly prepared, and should show no sign of reduction on heating.

Dilute the reagent with about three times its volume of water, heat almost to boiling-point, and add the glucose solution gradually, until maximal reduction is produced. The solution requires to be kept almost at boiling-point. The reduction is similar to that described under Trommer's test, a proportionally larger amount of the cuprous salt being, however, in the form of the oxide (red), owing to the greater alkalinity.

(c) BENEDICT'S TEST.

Add about 5 drops of glucose solution to 5 c.c. of Benedict's qualitative reagent¹ and boil for a few minutes. A yellow precipitate, which becomes red on more prolonged boiling, separates out. Sodium carbonate and sodium citrate replace in this test the caustic soda and sodium potassium tartrate of Fehling's solution.

(d) BARFOED'S TEST.

Barfoed's reagent is an acetic acid solution of cupric acetate, which is reduced by glucose to a slight extent, but not by maltose or lactose.

Add a small quantity of Barfoed's reagent to the glucose solution, and keep boiling until an opacity, due to presence of red granules, is perceptible in the fluid. Examine against a black background with bright light passing through the solution.

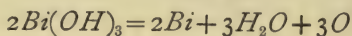
The red granules of Cu_2O are seen adhering to the sides of the tube.

(e) BOETTGER'S TEST.

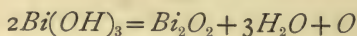
(a) *Add a few grains of subnitrate of bismuth and about double the quantity of sodium carbonate to the solution of glucose (about a quarter of a test-tube). After boiling for a short time, the bismuth hydrate becomes*

¹ See page 385 of Appendix.

reduced to metallic bismuth (?), or more probably bismuth dioxide (Bi_2O_3), the deposit acquiring a grey or black colour.



or



(Black).

A modification of this test, analogous to the Fehling modification of Trommer's test, and possessing the same advantages as the former, is often employed (Nylander's test).

[(β) Add about one part of the reagent¹ to five or more parts of the glucose solution, and boil for two to five minutes. A black precipitate gradually separates out on cooling.]

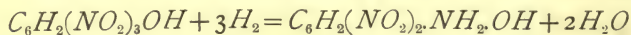
(f) SILVER TEST.

Take some ammoniacal silver nitrate solution² in a test-tube, add a few drops of glucose solution, and warm in water bath or over small flame. A mirror of metallic silver gradually forms.

In addition to reactions depending upon the reduction of metallic oxides, there are others which are often employed for the detection of glucose. Among these the following should be carried out:—

(g) PICRIC ACID TEST.

Add to 5 c.c. of glucose solution one-fourth volume of caustic soda solution, and one-fourth volume of picric acid (aqueous solution). On heating, a deep red colour is formed, due to the reduction of picric to picramic acid.



(Picric acid).

(Picramic acid).

[(h) Glucose reduces indigo blue to indigo white, and the latter can be re-oxidised by shaking up with air. Reduction can again be brought about by heating.

To a small quantity of the glucose solution, diluted with about ten times its volume of water, add a drop or

¹ See Appendix.

² Prepare this solution by adding a few drops of caustic soda to about 5 c.c. of the solution of silver nitrate, and sufficient ammonia to dissolve the precipitate of silver oxide

two of freshly prepared sulphindigotate of soda solution (just sufficient to colour the solution), make alkaline with Na_2CO_3 solution, and heat. The indigo blue is gradually reduced to a colourless solution, passing through intermediate coloured stages. Cool in water and shake up. The solution again becomes blue, and on heating is reduced.]

[A similar test can be carried out with a freshly prepared solution of potassium ferricyanide taking the place of the indigo blue. On heating the solution of glucose with Na_2CO_3 , the ferricyanide is reduced to ferrocyanide, the solution becoming almost colourless.]

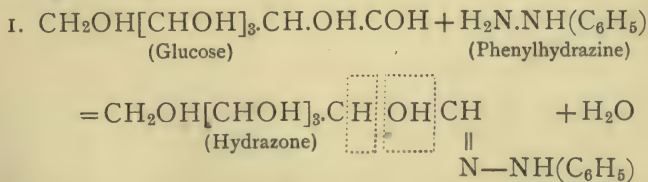
(i) MOORE'S TEST.

Add to the glucose solution about half its volume of KOH, and heat. The solution gradually acquires a yellowish-brown colour. Add some dilute sulphuric acid, and the odour of caramel becomes distinct.

(j) PHENYLHYDRAZINE TEST.

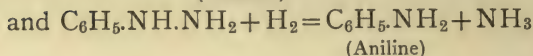
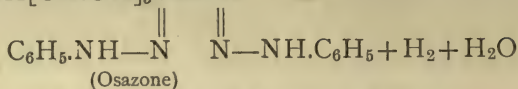
In carrying out this reaction, it is advisable to keep to definite proportions of the salts employed, and also to the temperature; because, if this be done, one can, from the time of the appearance of the osazone crystals and their amount, be almost certain of the sugar present, before the melting point is taken.

The stages in the osazone formation are shown in the following two equations:—



2. When a second molecule of phenylhydrazine enters, the H.OH is replaced by $=\text{N.NH.C}_6\text{H}_5$ to form an osazone, the hydrogen which is separated breaking up at once another molecule of phenylhydrazine to aniline and ammonia. Thus—

Hydrazone + phenylhydrazine



Take 50 c.c. of a 1 per cent. solution of water-free glucose, add 3 c.c. of a solution of phenylhydrazine in acetic acid¹ (40 grms. phenylhydrazine, 40 grms. glacial acetic acid in 100 c.c. water), and keep at a temperature as nearly as possible 100°² for twenty minutes; then cool. Note the time of appearance of a precipitate, and compare with lactose and maltose treated in same way. The yellow crystalline mass, which begins to form eight to ten minutes after the maximal temperature has been reached, and completely separates only after cooling, can be dried, and the melting point taken. The crystals should also be examined microscopically and drawn (see Fig. 10, Plate of Crystals, at end of book). They are very fine needle-like crystals arranged in sheaves.

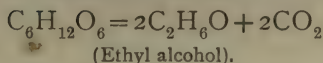
When only a small quantity of the solution of glucose is available this test may be carried out in the following way:—Add two or three drops of phenylhydrazine (or 0.15 to 0.2 gram. of phenylhydrazine hydrochloride) and two or three drops of 50 per cent. acetic acid (or 0.3 gram. of sodium acetate) to 5 c.c. of a 1 per cent. solution of glucose placed in a test-tube and heat on the water bath in the way already described.

(k) FERMENTATION.

Add a few particles of washed yeast to the glucose solution in an Einhorn's fermentation tube or in a test-tube, dipping into a mercury bath. Place in the incubator at body temperature.

¹ Or, 2½ grms. phenylhydrazine hydrochloride and 5 grms. sodium acetate may be dissolved in the same volume of glucose solution.

² The temperatures throughout the book are Centigrade.



Fermentation goes on best at a temperature of about 34° . The CO_2 which collects in the tube may be absorbed by passing in a small quantity of concentrated KOH.

It is advisable, when one is dealing with an unknown reducing body, to test in same way a control tube of yeast and water, and also one of yeast and a solution of glucose.

[(*l*) MOLISCH'S OR THE FURFUROL TEST.]

Take about $\frac{1}{2}$ c.c. of glucose solution, add a drop of a cold saturated solution of α -naphthol in methyl alcohol (must be free from acetone), then carefully pour into inclined test-tube about 1 c.c. of pure concentrated H_2SO_4 , so that it forms a well-defined lower layer. Where this comes into contact with the upper layer, a violet ring appears either immediately or after a very short interval (varying with different carbohydrates). Now, mix the fluids while the tube is kept cool in a stream of water; the colour becomes reddish-violet, and if it be examined quickly with the spectroscope, a small band will be seen between D and E, while the violet end will show total absorption.

This test is given by all carbohydrates, glucosides, and proteins which contain a carbohydrate radicle.

(*m*) POLARIMETRIC.

The solution to be examined must be free from other optically active substances (*e.g.* protein), and perfectly transparent. In order that the test may be of value in recognising a particular sugar, it is necessary, not only that the above conditions should be fulfilled, but also that the strength of the solution be known, so that the specific rotatory power may be calculated, when the rotation produced by a solution of known strength examined in a layer of definite length has been determined. The solution, also, should not be examined when freshly prepared, but after twenty-four hours have elapsed. Aqueous solutions of glucose contain two isomeric forms of the closed ring type known as α - and β -glucose.

These have been prepared by E. F. Armstrong from the corresponding methyl glucosides. The α - form has the specific rotatory power $+110^\circ$, and the β - form $+20^\circ$. A freshly prepared solution of glucose has a high specific rotation, since the sugar is largely present in the α - form. The optical rotatory power gradually falls on standing as part of the α - form is converted into the β - form. The aqueous solution of glucose, which has finally attained a constant specific rotatory power ($[a]_D = +52.5$), either on standing or after the addition of ammonia, is an equilibrated mixture of the two forms. An aldehyde or open chain modification may also be present.^{1, 2}

It is also advisable to note the effect of previous subjection to the fermentation process, and also the effect of boiling with dilute acid upon the rotatory power of the solution.

The method of carrying out an estimation of specific rotatory power with the Laurent polarimeter will be shown.

Make 10 per cent. and 20 per cent. solutions of glucose.

Fix the zero point with the light from the Na flame, passing through a 2-dm. tube filled with distilled water.

Now fill the 2-dm. tube with the 10 per cent. solution, and note the degree of rotation of the analyser required to again obtain the point of uniform dimness on the two sides. Repeat with the 20 per cent. solution.

The specific rotatory power represents the rotation of the plane of polarisation produced by a solution containing 1 gm. of solute per c.c., when examined in a layer 1 dm. long.

If one then determines what rotation a solution of known strength produces, when examined in a layer 2 dm. long, the specific rotatory power is easily calculated.

The specific rotatory power, as determined with the Na flame (the D line in the solar spectrum), is written $[a]_D$; the observed rotation with any strength and any length of layer l is written a . If the substance rotates to the right the +

¹ See "The Simple Carbohydrates and Glucosides" (pages 116-123), E. F. Armstrong. Longmans, Green & Co., 1912. (Latest edition, 1919.)

² Fischer, Irvine and others have recently described another variety of glucose known as " γ -glucose," in which the closed ring is present, as in ethylene oxide. " γ -glucose" appears to exist in two isomeric forms.

sign is inserted, if to the left the $-$. The temperature at which the observation is made should also be indicated by writing it above the letter D thus $[\alpha]_D^{20}$.

$$\text{Thus } [\alpha]_D = \pm \frac{a \times 100}{c \times l} \quad \begin{array}{l} c = \text{grms. per 100 c.c. solution.} \\ l = \text{length of tube in dms.} \end{array}$$

When the specific rotatory power of water-free glucose is calculated from a 10 per cent. solution, it is found to be 52.74° to the right, gradually, although only slightly, rising as the strength increases, and diminishing as the concentration falls.

2. Levulose or Fructose (1 per cent. solution).

This keto-hexose is not so commonly met with as the aldose, grape sugar. It gives the same reduction tests, undergoes fermentation, and furnishes the same osazone as glucose. It may be distinguished by the two following tests:—

[(a) POLARIMETRIC.]

Rotates plane of polarised light more to the left than glucose does to the right. $[\alpha]_D^{20} = -92^\circ$. The specific rotatory power is greatly influenced by temperature, becoming less negative as the temperature is raised.

Fill 2-dm. tube and examine.

[(b) SELIWANOFF'S TEST.]

Take a small quantity of the solution in a test-tube, add a few grains of resorcinol, then about one quarter of its volume of concentrated HCl. On heating, the solution acquires a deep red tint, then a brownish-red precipitate forms which can be dissolved in ethyl or amyl alcohols, the fluid then being of a deep red colour.

This test may also be carried out with Seliwanoff's reagent, prepared by dissolving .05 gm. of resorcinol in 100 c.c. of a mixture of equal parts of concentrated hydrochloric acid and water. Add about 10 drops of the solution of fructose to 5 c.c. of the reagent and heat gradually to the boiling point.

This test is given by all keto-hexoses and by carbohydrates which furnish these, e.g. cane sugar.

(c) This sugar may be obtained by hydrolysis of such a polysaccharide as inulin, or by the inversion of cane sugar.

[3. (d) Galactose.]

This aldo-hexose occurs as a constituent of lactose, raffinose, of some glucosides, of many gums, as the polysaccharide galactan or galactosan, and of complex compounds known as cerebro-galactosides, which have been isolated from the central nervous system.

(a) *It gives the same reduction tests as glucose, including Barfoed's test.*

(b) *It only very slowly undergoes alcoholic fermentation with yeast.*

(c) *On oxidation with dilute nitric acid, it yields about twice as much mucic acid as the same amount of lactose. This test is carried out in the way described under lactose.*

(d) *Phenylhydrazine test.*—A phenyl galactosazone may be prepared by the method described for glucose. The osazone crystallises in thick yellow needles, which are fairly soluble in boiling water. The solution should, therefore, be allowed to cool before the crystalline deposit is examined microscopically.

(e) *It has the specific rotatory power $+81^\circ$.*

(f) *It readily crystallises as rhombic plates on concentrating its aqueous solution by evaporation.*

The most characteristic reaction of this sugar is (c).

[4. (d) Mannose.]

This sugar is widely distributed in nature in the form of polysaccharides known as mannans or mannosans. The latter yield mannose when hydrolysed by dilute mineral acids. Its solutions show mutarotation, and the specific rotatory power of the equilibrated solution is $+14^\circ$.

(a) *It gives the same reduction tests as glucose.*

(b) *Phenylhydrazine test.*—(a) *When phenylhydrazine acetate is added to its solution in the cold and the mixture vigorously shaken, a precipitate consisting of colourless rhombic prisms separates out in a few minutes. This precipitate is the slightly soluble phenylhydrazone of mannose. The phenylhydrazones of all the other sugars are*

readily soluble in water. This test is therefore characteristic of mannose.

(b) *When the sugar is heated with phenylhydrazine acetate, it yields the same osazone as glucose.*

(c) *It readily undergoes the alcoholic fermentation when yeast is added to its solutions.*

II. PENTOSESES.¹ ($C_5H_{10}O_5$)

Although the hexoses are the most important sugars among the monosaccharides, there is another group which is of considerable physiological interest, namely, the pentoses, which have been discovered in the urine in different conditions.

They give the ordinary reduction tests, form osazones, but do not ferment. There are two reactions which they, and the carbohydrates which furnish them, give, and other carbohydrates do not. The only substance that does give these reactions, and may be confused with pentoses, is glycuronic acid. The method of recognising this acid will be given subsequently.

Gum arabic, which yields arabinose and galactose on hydrolysis, may be employed for the reactions, if xylose cannot be obtained.

Pine wood shavings also give the pentose reactions, owing to the presence of xylose.

The following are the two tests referred to:—

(a) PHLOROGLUCINOL REACTION.

Take a small quantity of distilled water in a test-tube, add an equal quantity of pure concentrated HCl, and then, into the acid fluid, which is kept warm over the flame, sprinkle phloroglucinol until a little remains undissolved. Add a very small quantity of gum arabic, and warm test-tube in water bath or well above the flame. Solution becomes cherry-red in colour, and gradually a precipitate settles out which may be dissolved out in amyl

¹ The pentose reactions may be shown to the class as a demonstration.

alcohol and examined spectroscopically (a band between D and E).

(b) ORCINOL REACTION.

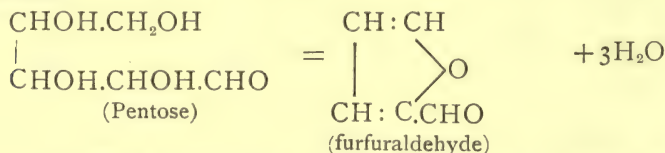
Carry out test exactly in same way as above, only substitute orcinol for phloroglucinol. The solution acquires a reddish-blue colour on warming, with an absorption band between C and D, near D. The colour rapidly changes from violet to blue, red, then green, and gradually a bluish-green precipitate separates out which dissolves in amyl alcohol, the alcoholic solution showing the above spectrum.

The colour reactions (a) and (b) are both dependent on the formation of furfuraldehyde.

(c) No fermentation occurs with pentoses.

Test some xylose solution with yeast.

(d) When any hexose is boiled with a fairly concentrated solution of a mineral acid, it yields amongst other products lævulinic acid¹ ($\text{CH}_3\text{CO}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{OH}$) and only traces of furfurol or furfuraldehyde; while pentoses, under the same conditions, are converted almost quantitatively into furfurol, no lævulinic acid being found amongst the products of decomposition.



(a) Boil vigorously a small portion of gum arabic, or preferably cherry gum with 10 times its bulk of dilute sulphuric acid (one volume of concentrated sulphuric acid to 5 or 6 volumes of water). After about a minute, hold a piece of filter paper impregnated with aniline acetate (1 drop of 50 per cent. acetic acid plus 1 drop of aniline) in the vapours from the test-tube. A carmine red colour due to the formation of a compound of aniline with furfurol is produced on the filter paper.

(b) Carry out the same test with glucose or starch or cane sugar instead of gum arabic. The reaction is

¹ Also written levulinic acid.

less distinct, especially with glucose and starch, and only occurs after prolonged boiling. Cane sugar gives a positive reaction fairly rapidly.

(c) The pentoses (xylose and arabinose) give all the reduction tests, including Barfoed's. Unfortunately, the difficulty of preparing the pure sugars prohibits their use for class work.

Gum arabic may be hydrolysed in the following way:—Place 100 c.c. of $N/2$ hydrochloric acid in a flask, heat to the boiling point, allow to cool slightly and add 5 grammes of gum arabic. Heat the solution on the water bath for 2 hours in a flask provided with a reflux condenser, then neutralise with sodium carbonate; and filter. This solution, which contains mainly galactose, and the pentose (arabinose) with smaller amounts of other sugars, will be found to give all the reduction tests, and the colour reactions for the pentoses. The presence of galactose may be recognised by the fact that, when three volumes of the solution are mixed with one volume of concentrated nitric acid, and the resulting solution concentrated to about one-sixth of its original volume on the water bath, mucic acid separates out.

When cherry gum is hydrolysed in the same way, it gives a larger yield of arabinose than gum arabic.

B. DISACCHARIDES ($C_{12}H_{22}O_{11}$).

These are condensation products of the hexoses, two molecules of the latter losing one of water.

I. Maltose (1 per cent. solution).

(a) Carry out some of the REDUCTION TESTS (a), (b), (c), (d), described on pp. 13 to 15.

All give positive results except (d).

Note the delay in the reduction in the other tests compared with glucose.

(b) Do MOORE'S TEST. Positive.

(c) *Form the PHENYLMALTOSAZONE, in the way described on p. 18, heating, however, for one and a half hours.*

It is much more soluble than the corresponding glucosazone, and only separates out from the fluid on cooling. Examine crystals microscopically and sketch them (*see Plate*).

Note the difference from the glucosazone ones in size and arrangement. The crystals are much thicker than those of glucosazone and are usually arranged in rosettes.

(d) *Put some of the solution, with yeast, in an Einhorn's tube, kept at 34°.*

It ferments.

(e) *Examine in POLARIMETER.*

Rotates to right, and specific rotatory power much greater than glucose. $[\alpha]_D^{20} = +137^\circ$.

Note the effect of boiling with dilute acid upon the rotatory power (same volume before and after boiling).

The fall in rotatory power is due to the hydrolysis of maltose to glucose.

The tests which distinguish it from glucose are the absence of reduction of cupric acetate (Barfoed's test), the solubility and crystalline form of the osazone, and the specific rotatory power.

2. Lactose (1 per cent. solution).

(a) *Carry out the reduction tests (pp. 13 to 17).*

All give positive results except (c).

(b) *Do MOORE'S TEST. Positive.*

(c) *Form the PHENYLLACTOSAZONE crystals, as described on p. 18.*

Like the maltosazone, those only separate out after cooling.

Examine them microscopically, sketch and compare their size and arrangement with those of maltose and glucose (see Plate). The crystals are arranged in circular clusters of fine wavy needles, radiating out from a dense centre.

(d) Put some of the solution with yeast in an Einhorn's tube, kept at 34° .

Does not ferment.

(e) Examine in POLARIMETER before and after boiling with dilute HCl for about half an hour, the volume being made up to the original.

The rotation to the right is greater after the hydrolysis, owing to the formation of galactose $[\alpha]_D = +81^{\circ}$ along with glucose, while the $[\alpha]_D$ of lactose is only 52.53° . (Boiling with dilute acid produces practically no effect on the rotatory power of glucose, and diminishes that of maltose owing to the formation of glucose.)

[(f) FORMATION OF MUCIC ACID.¹]

Take 12 c.c. of HNO_3 (sp. gr. 1.15) in a porcelain basin, add 1 grm. of lactose, and evaporate down on water bath, stirring continuously, until about one-third of the fluid remains. A gritty deposit (just like sand) separates out, and if 2 c.c. of water be added to the acid fluid, the mucic acid can be filtered after twenty-four hours' standing, and the amount weighed.

Lactose and galactose both furnish mucic acid, the former also saccharic acid, while from cane sugar, maltose, glucose, starch, and dextrin, treated in the same way, no such gritty deposit separates out, as saccharic acid alone is formed.

This is a good test for lactose, and can be quickly carried out.

As distinctive tests for lactose, the most important are—

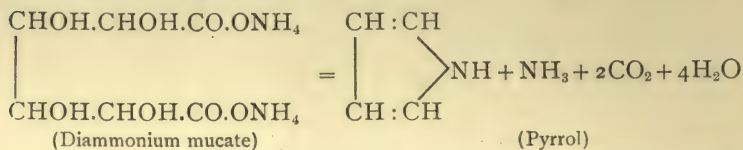
(a) The non-fermentability with ordinary yeast; (β) the formation of mucic acid; (γ) the marked increase in dextro-rotation on hydrolysis with acids; (δ) the time of formation and form of the osazone crystals.

[(g) PYRROL TEST.]

Place a small quantity of mucic acid in a dry test-tube, add a drop or two of strong ammonia solution, and heat on the water bath until all the free ammonia has been driven off and the residue has become dry. Then heat the test-tube in a bunsen flame, and hold a match previously dipped in concentrated hydrochloric acid in the vapours

¹ This test should be done in the draught chamber.

from the test-tube. The part of the match which had been dipped in hydrochloric acid becomes deep red in colour. This colour reaction is given by pyrrol and many of its derivatives, e.g. indol.



Diammonium saccharate also yields pyrrol when heated strongly.

3. Cane Sugar (1 per cent. solution).

(a) Make the solution alkaline with KOH, and add drop by drop CuSO_4 , shaking after the addition of each drop. The solution gradually becomes deeper blue in colour, and finally will dissolve no more of the hydrate. No reduction, however, occurs on heating, if the sugar be pure.

It dissolves $\text{Cu}(\text{OH})_2$, giving a deep blue-coloured solution.

(b) When pure, this sugar gives none of the reduction tests.

Do TROMMER and FEHLING.

(c) Add a few drops of HCl to some of the solution in a test-tube, and boil for a few minutes. Pour a little into a test-tube, make alkaline, add Fehling, and boil. Distinct reduction to cuprous hydrate and oxide is now apparent, due to the formation of so-called invert sugar, a mixture of glucose and levulose.

(d) Run carefully down the side of the test-tube, containing cane-sugar solution, conc. H_2SO_4 . Charring occurs, first at the line of contact and then, on shaking, throughout the fluid.

(Negative, or merely very slight charring, with glucose, lactose, and maltose.)

(e) Does not give MOORE'S TEST (see p. 17).

(f) A solution of cane sugar and one of invert sugar may be examined in the polarimeter.

Cane sugar rotates the plane of polarised light to the

right ($[\alpha]_D^{20} = +66.5^\circ$), while invert sugar (a mixture of equal quantities of glucose and levulose or fructose) is lævo-rotatory, in virtue of the fact that levulose rotates more to the left than glucose to the right.

Before examining in the polarimeter any of the acid hydrolysed solutions, neutralise the acid almost completely (not completely, in order to avoid increased depth of tint of the fluid).

(g) *When treated with PHENYLHYDRAZINE, as previously described, no osazone is formed.*

(h) *Fill an Einhorn's tube with the solution, add yeast, and keep at 34° .*

Fermentation occurs, due to the fermentation of invert sugar, produced by means of the inverting enzyme in the yeast.

(i) This sugar also gives Seliwanoff's test [test (b) for levulose], as it furnishes levulose on hydration.

The characteristic tests for cane sugar are the charring with H_2SO_4 , the true rotatory inversion on hydrolysis, the absence of the reduction and osazone tests.

C. POLYSACCHARIDES ($C_6H_{10}O_5$)_n.

This class contains bodies of higher molecular weight than the other two. They are polymers of $C_6H_{12}O_6$ which has lost one molecule of water.

1. Starch (1 per cent. solution).

(a) THE IODINE TEST.

Add a few drops of dilute iodine solution; blue iodide of starch is formed. Pour a small quantity into another test-tube, add a drop or two of KOH; the blue iodide is decomposed and the colour disappears, reappearing when the solution is made slightly acid with HCl. Heat the iodide of starch solution in the other tube; colour disappears, reappearing on cooling.

(b) Does not reduce metallic oxides in alkaline solution nor is the precipitate of cupric hydrate dissolved.

Do TROMMER'S or FEHLING'S TEST.

(c) ¹ Take some starch solution in a porcelain basin or flask, make distinctly acid with HCl by adding 4 to 5 c.c. of the concentrated acid to each 100 c.c. of the solution, and boil. The opacity of the original solution gradually diminishes, and hydration goes on gradually to glucose. If the solution be tested with iodine, as the hydrolysis is proceeding, the blue compound soon fails to make an appearance, due to the transformation of starch to dextrin and dextrose. Pour some of the fluid into a test-tube after about fifteen minutes' boiling, make alkaline, add Fehling, and boil; reduction now occurs. Some of this glucose solution may be kept for polarimetric examination.

(d) Test in Einhorn's tube with yeast. No fermentation.

The same holds for all the polysaccharides.

Starch solution can be easily recognised by its blue iodide compound, the hydration of a non-reducing carbohydrate to glucose, and also the physical characters of the solution—a thin, opalescent mucilage.

2. Dextrin (1 per cent. solution).

(a) Add a drop or two of dilute iodine solution to some of the dextrin. A reddish-violet to reddish-brown colour appears, due to formation of iodide of dextrin. This colour is discharged by heating (reappearing on cooling) and by alkalies (reappearing on addition of acid).

The difference in colour of the iodide compounds of erythro-dextrins depends probably either upon admixture with soluble starch or upon the type of erythro-dextrin present.

(b) Do TROMMER'S TEST.

The cupric hydrate is only slightly dissolved, if the dextrin be pure; and, on heating, usually some reduction occurs, probably due mainly to admixture with maltose or glucose. Possibly even pure dextrin reduces CuO, as it is said to form

¹ The hydration of starch and the other polysaccharides should be carried out before the class meets.

an osazone, and therefore must contain the group upon which reduction depends. The reduction, however, which occurs with pure dextrin solutions is very slight.

(c) *Boil solution with dilute acid; glucose is formed, and thereby the amount of reduction is greatly increased. Make alkaline, and boil with Fehling's solution.*

¹(d) *Add some basic lead acetate to the solution. No precipitate appears.*

[(e) *Examine solution in polarimeter before and after hydrolysis.*]

After hydration to glucose there is a great fall in dextro-rotatory power.

This solution is characterised by its transparency (compared to starch), the colour of the iodide compound, and the non-precipitability with basic lead acetate.

3. Glycogen (1 per cent. solution).

Note the characteristic opalescence.

(a) *Add a few drops of iodine solution to a portion of the solution in a test-tube, reddish-brown coloration appears, due to formation of iodide of glycogen. Heat and alkalies have the same effect as with the iodides of the other two polysaccharides. Test their effect.*

(b) *Make some of the solution alkaline, add a few drops of CuSO_4 , and shake. The cupric hydrate is not dissolved. On heating, no reduction occurs, if the solution be free from glucose.*

(c) *Take some of the fluid in a porcelain basin, acidify with HCl , and boil for about a quarter of an hour; glucose is gradually formed.² Render the acid fluid alkaline with KOH , add Fehling's solution, and boil. Reduction to cuprous oxide occurs.*

(d) *Add some basic lead acetate to the solution; the glycogen is precipitated.*

¹ Starch, glycogen, and certain dextrans of high molecular weight, e.g. amylo-dextrin, are precipitated by basic lead acetate. This test is, therefore, not entirely satisfactory. Further, lead yields insoluble salts with many inorganic anions.

² It is advisable to show how this hydrolysis is carried out, using a sufficient quantity to supply all the members of the class.

This solution is characterised by its physical characters, its reaction with iodine, and its precipitation by basic lead acetate.

"SALTING OUT" POLYSACCHARIDES.

It is very important to remember that these polysaccharides (the majority at least) may be precipitated by saturating their solutions with such salts as $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 , and MgSO_4 , and hence may lead one astray in testing for proteins. Starch, glycogen, and probably all the dextrans, except achroödextrin, are precipitated. The precipitate therefore obtained on "salting out" a fluid should always be tested for polysaccharides, by dissolving in water and testing with iodine.

As an example of this, starch¹ may be tested.

Saturate solution with $(\text{NH}_4)_2\text{SO}_4$ crystals, filter, wash precipitate with saturated $(\text{NH}_4)_2\text{SO}_4$ solution, then dissolve it in water and test with iodine.

The sugars are not precipitated in the same way by neutral salts, and hence this method may be employed for separation of those carbohydrates.

Alcohol of different strengths may also be used for separating the different polysaccharides.²

[GLYCURONIC ACID.]

This acid is of importance because it occurs as a pathological constituent in urine, always in the paired form—*e.g.* with chloral hydrate as urochloralic acid. In this form it is lævo-rotatory, while as the free acid it is dextro-rotatory.

- (a) *It gives all the ordinary reduction tests, e.g. Trommer, Fehling, Boettger. Test.*
- (b) *It does not ferment. Test in Einhorn tube.*
- (c) *It gives the phloroglucinol test for pentoses (see p. 23). Test.*

¹ Starch may be almost entirely precipitated out of a solution by half saturation with $(\text{NH}_4)_2\text{SO}_4$, while dextrin as a rule requires complete saturation.

² Erythrodextrin is precipitated in a solution containing about 70 per cent. alcohol, achroödextrin in one of 90 per cent., and glycogen in about 60 per cent.

(d) *Also gives the orcinol pentose reaction (see p. 24).
Test.*

(e) In order to distinguish it from pentose, one of the best methods is to separate the acid in the form of the p. bromphenylhydrazone.

Take about 50 c.c. of glycuronic acid solution in a porcelain basin, add 1 grm. of hydrochloride of p. bromphenylhydrazine, and rather more sodium acetate. Bring mixture to boiling-point, and then keep on water bath for about fifteen minutes, when a mass of fine yellow crystals begins to appear. Cool. Filter off liquid. Wash crystals with absolute alcohol, in which they are insoluble. This hydrazone, unlike the p. bromphenylosazones, formed from sugars in the same way, is quite insoluble in absolute alcohol. Also, when dissolved in pyridin-absolute alcohol, it shows a much greater rotatory power than any of the latter.

Note.—The tests for glycuronic acid may be carried out with a yellow pigment, Indian yellow, which mainly consists of a magnesium salt of a paired glycuronic acid—euxanthinic acid. The best way to prepare it is as follows:—Mix up the powder with a little water, wash with dilute HCl, filter, wash away excess of HCl with water. Dissolve the insoluble material (euxanthinic acid) in hot alcohol, cool; crystals separate out. These may be recrystallised. This paired acid may be examined polarimetrically and chemically, or it may be split up into euxanthon and glycuronic acid, mixing up 1 grm. with 200 c.c. water, and digesting for about one hour at 120° to 125° in autoclave. Filter. Evaporate down filtrate to thin syrup, and allow the glycuronic acid to crystallise out (the anhydride). These tests may be carried out with this body.

Or, glycuronic acid may be prepared from urochloralic acid, obtained from dog's urine after chloral hydrate has been given (Drechsel).

APPENDIX TO CHAPTER ON CARBOHYDRATES

CONTENTS

- I. The polyhydric alcohols. Types—1, glycerol; and 2, mannitol.
 1. Glycerol. Properties of glycerol and some products of its oxidation.
 2. Mannitol. Preparation, properties, and some products of its oxidation.
- II. Preparation of the aldohexoses. 1, Glucose; and 2, galactose.
- 3, Preparation of the ketohexose,—fructose.
- III. Oxidation of glucose to saccharic acid, and the preparation of acid potassium saccharate.
- IV. Preparation of lævulinic acid from cane sugar. Some properties of lævulinic acid.
- V. Preparation of a disaccharide,—maltose.
- VI. 1, Preparation of a salt of an amino-hexose, glucosamine or chitosamine hydrochloride; 2, properties of glucosamine hydrochloride.
- VII. Preparation of the enzymes. 1, Emulsin; and 2, invertase.
- VIII. Preparation and properties of some glucosides. 1, Amygdalin; 2, salicin; and 3, phloridzin.
- IX. Preparation and properties of some polysaccharides.

I. Polyhydric Alcohols. Types—1, Glycerol; and 2, Mannitol.

1. **Glycerol** may be prepared from the products of saponification of neutral fats. The liquid remaining after separation of the fatty acids is neutralised, concentrated to a syrup, preferably at a pressure of about 15 mm. Hg, and the glycerol extracted with a mixture of three parts absolute alcohol and one part ether. The ether and alcohol are distilled off from the filtered extract, and the residue of impure glycerol purified by distillation under reduced pressure (10-15 mm. Hg). Pure glycerol is so readily obtainable that its preparation in the laboratory does not repay the time spent on its separation.

PROPERTIES.—Pure glycerol forms a thick colourless syrup of sp. gr. 1.265 at 15°, of neutral reaction, and of sweet taste. When kept

at a temperature below 0° it gradually separates as crystals, which melt at 17° .

(1) Heat a small quantity of glycerol in a dry test-tube. It ultimately boils (b.p. 290°) without obvious decomposition.

(2) It is miscible with alcohol and water in all proportions; but is insoluble in ether.

(3) Its power of dissolving cupric hydrate in alkaline solution has already been described.

(4) Acrolein test (see chapter on fats).

(5) Add a drop or two of methyl orange solution to six drops of glycerol previously mixed with 5 c.c. of water in a test-tube. Similarly add a drop or two of methyl orange solution to 5 c.c. of a cold saturated solution of boric acid. Mix half of the glycerol solution with half of the boric acid solution in a third test-tube, and compare the colours of the solutions in the three test-tubes. Note the deep red colour of the fluid in the third test-tube as compared with the yellow or orange colour of the solutions in the other two. The acidity of boric acid solutions is greatly increased by the addition of glycerol. The effect appears to be due to the formation of a complex acid of much higher dissociation constant than boric acid. The addition of mannitol raises the acidity of boric acid solutions to a still greater degree than glycerol. The aldohexoses produce a similar but less marked increase of acidity.

(6) Dip a borax bead fixed in a loop of platinum wire into a solution of glycerol, and bring the bead close to the border of the lower part of a non-luminous bunsen flame. The flame is coloured green by the glycerol-borate.

(7) PRODUCTS OF THE OXIDATION OF GLYCEROL.

Oxidation by hypobromites (E. Fischer).

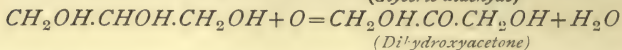
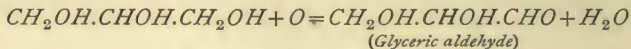
Dissolve 10 grms. of glycerol and 35 grms. of crystalline sodium carbonate in 60 c.c. of water. Cool the solution to about 10° , add 15 grms. of bromine (about 5 c.c.), and allow the solution to remain at room temperature for half an hour. The solution is then rendered slightly acid with hydrochloric acid, freed from bromine by passing sulphur dioxide through it, and accurately neutralised with caustic soda. A colourless syrup which dissolves readily in alcohol and ether is thus obtained. It gives the reduction tests, reducing Fehling's solution in the cold, and undergoes the alcoholic fermentation, when freshly prepared. It also yields a crystalline osazone. On heating, it becomes brown and yields a caramel odour (E. Fischer).

The oxidation of glycerol may be carried out on a small scale by the following method:—Dissolve two drops of glycerol and 0.5 gm. of crystalline sodium carbonate in 5 c.c. of water. Cool the solution, add one drop of bromine, shake thoroughly, and allow the fluid to stand in the cold for half an hour. Then acidify the solution slightly with hydrochloric acid, extract any free bromine by shaking up with about 1 c.c. of carbon tetrachloride, allow the latter to settle to the bottom of the test-tube, and transfer the aqueous solution

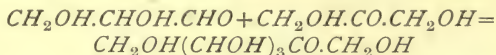
with a pipette to another test-tube. Carry out the following tests with portions of this solution:—

(a) Add a few drops (5 or 6) of copper sulphate solution, and render alkaline with caustic soda. A clear blue solution, from which yellow cuprous hydrate soon separates out in the cold, is obtained.

The glycerol has been oxidised to glycerose, a mixture of keto- and aldo-triose known respectively as dihydroxyacetone, and glyceric aldehyde.



When dilute alkali (1 per cent. caustic soda) is allowed to act for 4 to 5 days at 0° on glycerose, the latter undergoes the "aldol" condensation resulting in the formation of a mixture of hexoses known as acrose (α and β), from which E. Fischer has prepared synthetically a large number of hexoses.¹



(b) Carry out the resorcinol test in the way already described (p. 21 (b)). A red coloured solution is obtained, from which the red colouring matter may be extracted with amyl-alcohol.

2. **Mannitol.**—Preparation. Mannitol may be most readily obtained from manna, the dried sap of an ash (*fraxinus ornus*) which grows in the East.

50 grms. of manna are boiled for about half an hour on the water bath with 250 c.c. of 90 per cent. alcohol in a flask provided with a reflux condenser. The alcoholic solution is filtered, while still hot, through a funnel with hot water jacket. The residue of manna in the flask is again boiled with about 150 c.c. of fresh alcohol, and the resulting solution filtered. The united filtrates are allowed to cool in a stoppered flask. Fairly pure mannitol rapidly separates out as fine needle-shaped crystals. The alcoholic solution is left overnight in the ice-chest, the crystalline deposit is filtered off with the aid of suction by means of the water-pump through a Buchner funnel, and purified by recrystallisation from 70 per cent. alcohol, to which some animal charcoal may be added. The recrystallised mannitol is finally filtered off, partially dried by suction, and then in an evacuated exsiccator over anhydrous calcium chloride. The yield is about 20 grms.

PROPERTIES.

(1) Heat a small portion in a dry test-tube. The substance melts to form a colourless liquid, which becomes brown and chars on further heating. The melting point, determined in a capillary tube, is 165° - 166°.

¹ See "The Simple Carbohydrates and Glucosides," by E. F. Armstrong, pages 89-91.

- (2) When pure, it leaves no residue when heated on platinum foil.
 (3) Its aqueous solution is neutral and sweet.
 (4) Its alkaline solutions dissolve cupric hydrate; but do not reduce it even on heating. If reduction does occur, the mannitol should be recrystallised from 70 per cent. alcohol to free it completely from reducing sugars.
 (5) Dissolve a little mannitol in a few c.c. of warm 70 per cent. alcohol, and examine with the microscope the crystals, which separate out on cooling.

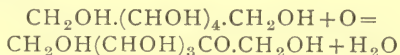
(6) PRODUCTS OF THE OXIDATION OF MANNITOL.

(a) Oxidation with sodium hypobromite (*E. Fischer's method slightly modified*).—Dissolve in a test-tube 1 gm. mannitol and 2.4 grms. crystalline sodium carbonate in 8 c.c. of water, cool the solution, add 5 drops of bromine, shake thoroughly, and allow the solution to remain at room temperature for half an hour. Then acidify the solution with dilute hydrochloric acid, and extract excess of bromine with carbon tetrachloride. Transfer the aqueous solution with a pipette to another test-tube, and divide the fluid into two parts.

(a) Test one portion by Trommer's test. Reduction occurs on heating.

(β) Carry out the resorcinol test (see cane sugar, p. 29 (i)) with the other portion. The colour reaction is well marked.

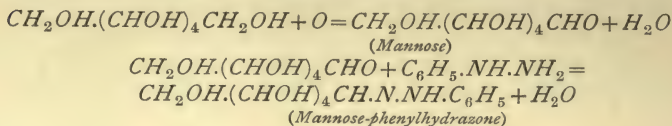
The chief product of the oxidation of mannitol under the conditions of the experiment appears to be fructose. The writer was unable to detect any mannose.



(b) Oxidation with dilute nitric acid at 40° to 45°,—formation of mannose (*E. Fischer*).—Dissolve 3 grms. of mannitol in 20 c.c. of water, add 10 c.c. of concentrated nitric acid, and heat on the water bath for 4 to 5 hours at a temperature of 40° to 45° (temperature of the reaction fluid, not of the water bath). A visible reaction with development of gas then usually occurs. Take about 1 c.c. of the fluid, render the sample faintly alkaline with sodium carbonate then render acid with acetic acid, cool, and add a little phenylhydrazine dissolved in dilute acetic acid. If a dense crystalline precipitate separate out after the fluid has been vigorously shaken and allowed to stand for about 20 minutes, the reaction may be regarded as complete. If no precipitate form, the heating of the original solution should be continued, and samples tested at intervals of 20 minutes in the way just described until a positive result is obtained. The oxidation is frequently not complete until the heating has been continued for 6-7 hours. Great care has to be taken to prevent any rise of temperature above 45° during the oxidation. If this occurs, or the oxidation be too prolonged, the mannose, which is first formed, undergoes further oxidation to mannonic acid, or even to manno-saccharic acid.

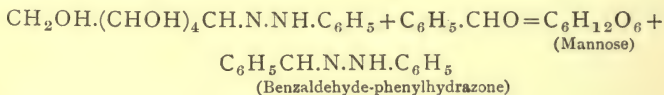
The fluid is finally cooled, rendered faintly alkaline with sodium carbonate, then acidified with acetic acid. 1 gm. of phenylhydrazine

dissolved in dilute acetic acid is then added, the solution is well shaken and allowed to stand for some hours at room temperature. The sparingly soluble phenylhydrazone of mannose which gradually separates out, as a light yellow coloured precipitate, is filtered off through hardened filter-paper, using the filter-pump, washed with cold water, then with warm acetone, and partially dried by suction. The precipitate is then dissolved in boiling water, and filtered while hot. On cooling, the phenylhydrazone of mannose crystallises out from the filtrate as colourless rhombic prisms or plates.



Mannose may also be prepared by oxidising mannitol with hydrogen peroxide in the presence of ferrous sulphate (Fenton and Jackson). The most productive and convenient method of preparing it is by the acid hydrolysis of the vegetable ivory nut (*phytelephas macrocarpa*) (Reiss).

Pure mannose may easily be prepared from its hydrazone by decomposing the latter by heating for half-hour on the water bath with benzaldehyde dissolved in 50 per cent. alcohol. The precipitate of the phenylhydrazone of benzaldehyde is filtered off, and the filtrate, after being freed from excess of benzaldehyde by extraction with ether, is decolorised with animal charcoal and concentrated to a syrup, from which mannose gradually crystallises out. Further details are omitted, since this separation is only advisable when a larger quantity of the phenylhydrazone of mannose is available than that yielded in the foregoing preparation.

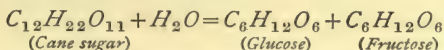


II. Preparation of other Aldohexoses.

1. Glucose.—Preparation from cane sugar (Soxhlet).

750 c.c. of 90 per cent. ethyl alcohol and 30 c.c. of concentrated hydrochloric acid (sp. gr. 1.19) are heated to 45°–50° on the water bath. 250 grms. of finely powdered cane sugar are gradually added in small quantities, the liquid being thoroughly shaken after each addition until the sugar has dissolved. When 250 grms. of cane sugar have been dissolved, the warm fluid is filtered and allowed to cool. On standing for a day or two in the ice chest, a crystalline deposit of anhydrous glucose separates out. The crystallisation may be hastened

by inoculating the alcoholic solution with a small quantity (about 0.5 grm. or less) of anhydrous glucose. The crystals are finally filtered off, washed with alcohol, and dried over anhydrous calcium chloride in an evacuated exsiccator. The sugar may be purified by dissolving it in a small quantity of water on the water bath, then adding warm absolute alcohol until the fluid begins to become turbid. The warm solution is then filtered and the filtrate allowed to cool. Glucose crystallises out, the separation being hastened by shaking the alcoholic solution while cooling.

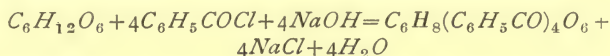


The fructose which is also formed remains in solution in the alcohol.

Glucose may also be prepared by heating one part of starch with 10 parts of 2 per cent. hydrochloric acid on the water bath for about 3 hours. The yield is over 90 per cent. of that theoretically possible.¹

Preparation of a benzoic acid ester of glucose. Since glucose contains five hydroxyl groups, it yields esters with a number of acids. The tetrabenzoyl ester may be prepared in the following way (Baumann):—

Place 1 grm. of glucose (1 mol) in a stoppered bottle, dissolve in 20 c.c. of water, add 40 c.c. of 10 per cent. caustic soda (18 mols) and 6 c.c. of benzoyl chloride. Shake the mixture vigorously. A white substance, which mainly consists of the tetrabenzoyl compound, gradually separates. It may be crystallised from alcohol.



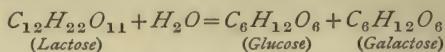
The most important reactions of glucose have already been given in Chapter II.

2. Galactose.

Preparation—50 grms. of lactose are heated for 4 hours on the boiling water bath with 500 c.c. of 2 per cent. sulphuric acid. The liquid is then freed from sulphuric acid by the addition of excess of calcium carbonate, filtered, and the filtrate concentrated to a thin syrup on the water bath. The syrup is then left in the ice chest for two or three days. Galactose separates out in crystalline form (as colourless rhombic plates). Part of the syrup is poured off and the sediment is transferred by washing with 80 per cent. alcohol on to a filter-paper placed in a Buchner porcelain funnel. The crystalline precipitate of galactose is partially dried by suction with the water pump, washed twice or thrice with 80 per cent. alcohol, and dried in vacuo in an exsiccator. The galactose may be purified by adding the finely powdered sugar to warm 70 per cent. alcohol so as to form a saturated solution. The warm alcoholic solution after being filtered is allowed to cool. Crystals of galactose separate out. Decolorisa-

¹ See "Practical Physiological Chemistry," by S. W. Cole, 1919, p. 105. Publishers: W. Heffer, Cambridge.

tion with animal charcoal is usually unnecessary. Galactose crystallises more readily than the other hexoses.



The other product of hydrolysis (glucose) remains dissolved in the syrup and in the 80 per cent. alcohol used for washing the galactose.

3. Preparation of the keto-hexose, fructose from cane sugar.

60 grms. of cane sugar are dissolved in 300 c.c. of water, 5 c.c. of *N*/1 hydrochloric acid are added, and the solution is made up to about 500 c.c. in a flask of about 750 c.c. capacity. The solution is then raised to the boiling-point, and placed on the boiling water bath for about 30 minutes. The solution is then cooled, preferably finally with ice and water. It is advisable that the temperature of the solution should not be higher than 10° C. Then add 200 c.c. of a 10 per cent. suspension of calcium hydrate in water. Shake vigorously for two to three minutes (not longer), preventing any rise of temperature by cooling with tap water, then filter rapidly with the aid of suction through filter-paper placed in a Buchner funnel. The calcium compound of fructose, being only slightly soluble in cold water (1 in 137), rapidly separates out from the filtrate as fine, colourless rhombic prisms. The filtrate should be left in the ice chest for 8-12 hours to ensure more complete separation of the calcium compound. The crystalline deposit of calcium fructosate is then filtered off, washed with ice-cold water, and partially dried by suction with the water pump. Small portions of it may be removed with a spatula and used for the following tests:—

(1) Suspend a small quantity of the calcium compound in water and heat gradually. The substance dissolves, and the resulting solution rapidly becomes yellow, and then brown, owing to the formation of humin substances (Moore's test). The colour change is hastened by the addition of caustic soda. If the coloured solution be acidified with dilute sulphuric acid and heated, it is partially decolorised, and the so-called caramel odour develops. Fructose is very readily decomposed by the action of alkalies.

(2) Suspend another larger quantity of the calcium compound in water, and render slightly acid by the addition of dilute acetic acid. Heat a part of this solution, no change in colour is produced.

(3) The solution in dilute acetic acid may be used for all the tests for fructose already described (e.g. Fehling, Barfoed, etc.), since the presence of calcium acetate does not appreciably interfere with the reactions.

Pure fructose may be prepared from the calcium compound by suspending the latter in ice-cold water and adding sufficient oxalic acid to precipitate the calcium. The precipitate of calcium oxalate is filtered off, and any free oxalic acid in the filtrate removed by the addition of a little calcium carbonate. The solution is again filtered, and the filtrate concentrated by distilling off the water *in vacuo* at as low a temperature as

possible. It is difficult to obtain crystals of fructose from the syrupy residue. References to the literature on the subject will be found in textbooks dealing specially with carbohydrates.

III. Oxidation of Glucose to Saccharic Acid, and the Preparation of Acid Potassium Saccharate.

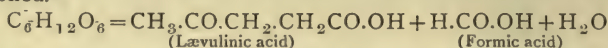
50 grms. of starch are gradually added to a mixture of 400 c.c. water and 92 c.c. of concentrated nitric acid (sp. gr. 1.41) previously heated on the boiling water bath. The starch is first hydrolysed yielding glucose, which later undergoes oxidation to saccharic acid. The solution, which should be frequently stirred, is concentrated on the water bath (in a fume cupboard) to a syrup, which is diluted with a little water and again evaporated. The evaporation is stopped as soon as the syrup commences to become brown. The syrup is dissolved in 150 c.c. of water, neutralised with a concentrated solution of potassium carbonate, then mixed with 25 c.c. of 50 per cent. acetic acid and concentrated to a volume of about 80 c.c. on the water bath, preferably in vacuo. The concentrated solution is left in the ice chest, and should be stirred occasionally. The crystals of acid potassium saccharate, which separate out, are filtered off with the aid of the suction pump, washed with a little cold water, and dissolved in as little boiling water as possible, to which a little animal charcoal is added. The warm solution is filtered, and after concentration in vacuo yields colourless rhombic needles of acid potassium saccharate (solubility in cold water, 1.1 per 100).

Free saccharic acid is exceedingly soluble in water, in marked contrast to the stereoisomeric mucic acid yielded by galactose under similar conditions. Mannose yields mannosaccharic acid on oxidation with dilute nitric acid. The latter acid is readily soluble in water, and does not form a sparingly soluble acid potassium salt. These characteristics distinguish it from mucic acid on the one hand, and from ordinary saccharic acid on the other.

IV. Preparation of Lævulinic Acid from Cane Sugar.

All hexoses and substances, such as the nucleic acid of the thymus containing them in complex combination, yield lævulinic acid when boiled with mineral acids—e.g. 7-10 per cent. hydrochloric acid, while pentoses yield no lævulinic acid under these conditions. The yield of lævulinic acid from ketohexoses is greater than from aldohexoses. Large quantities of dark brown and black so called humin substances are also produced, as well as formic acid. When cane sugar is boiled with

7 per cent. hydrochloric acid, it first undergoes inversion, and the glucose and fructose are then converted into the substances already mentioned.



Place 50 grms. of cane sugar in a flask of 400-500 c.c. capacity, add 200 c.c. water and 50 c.c. of concentrated hydrochloric acid. Attach a reflux condenser to the flask, and heat the fluid on the water bath for 20 hours. Filter off the humus material on hardened filter-paper placed in a Buchner funnel and extract the humus residue with 50 c.c. of warm water. Add a solution of caustic soda containing about 15 grms. to the united filtrates and concentrate the solution on the water bath to about one-fourth of its original volume. The caustic soda is added in order to neutralise the greater part of the free hydrochloric acid. Extract the concentrated filtrate three or four times with 50 c.c. portions of ether. Transfer the ethereal solution to a flask and distil off the ether on the water bath. The residual liquid may be transferred to a small distillation flask (about 50 c.c.), distilled on an oil bath at a pressure of 15 to 20 mm. Hg, and the fraction boiling at 140° to 160° collected in a receiving distillation flask. When this fraction is left in the ice chest for 24 hours, the greater part of the lævulinic acid (m.p. 32.5°) crystallises out. This method may be adopted for the isolation of lævulinic acid, when a larger quantity of cane sugar (about 200 grms.) is taken than in the foregoing preparation.

The following is an alternative method of identifying lævulinic acid by means of certain reactions. Wash out the residue, remaining after the ether has been distilled off, with a little water into a porcelain basin, and heat for some time on the water bath to drive off formic acid.

(a) Transfer a little of the solution to a test-tube, add a little iodine dissolved in an aqueous solution of potassium iodide, and render the solution alkaline with caustic soda. A precipitate of iodoform separates out.

(b) Transfer another small quantity to a second test-tube, add a little fresh sodium nitro-prusside solution, and render alkaline with caustic soda. A dark red colour develops, which becomes fainter but is not discharged on acidifying with acetic acid.

(c) The remainder of the solution is heated with excess of zinc oxide and some animal charcoal, filtered while hot, concentrated by evaporation, and allowed to cool. The zinc salt of lævulinic acid, which crystallises out as colourless needles, is filtered off with the aid of suction through a filter-paper placed on a perforated porcelain plate. The precipitate is washed with a little alcohol and ether, dissolved in about 50 c.c. of water and mixed with a little more than the equivalent amount of silver nitrate, heated nearly to the boiling-point, to bring into solution the silver lævulinate which separates out, and the resulting solution is filtered while still warm. On cooling, the silver salt separates out in the form of characteristic hexagonal plates, which yield 48.4 per cent. of silver on incineration.

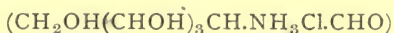
V. Preparation of a Disaccharide,—Maltose.

Rub up 100 grms. of starch with sufficient water to make a uniform paste and add gradually to 400 c.c. of water placed in a porcelain basin and previously raised nearly to the boiling-point, finally washing out the last traces of starch from the mortar with a little cold water. Heat on the water bath until an opalescent solution is obtained. Cool to 60°, and add 50 c.c. of an aqueous extract of malt, previously prepared by extracting 10 grms. of finely ground malt with about 50 c.c. of water for about an hour at 40° to 45°. Keep the temperature of the liquid at 55° to 60° for two hours, then boil and filter. The filtrate is then concentrated to a syrup either in a porcelain basin on the water bath, or preferably by distilling off the water in vacuo. Portions of the syrup are then boiled in a flask with reflux condenser with 90 per cent. alcohol, which dissolves the maltose, leaving the dextrins undissolved, and the filtered alcoholic extracts are united and concentrated to a thick syrup by distilling off the alcohol. The syrup is then transferred to a porcelain basin and placed in an evacuated exsiccator over anhydrous calcium chloride. Maltose gradually separates out as fine needles in the course of a few days. If crystallisation is delayed, it may be hastened by adding a little pure maltose to the syrup. The crystalline mass is then rubbed up with a little methyl-alcohol so as to form a paste, transferred to filter-paper placed on a perforated porcelain plate and partially dried by suction with the aid of the filter pump and washed twice with a little methyl-alcohol. This product is sufficiently pure for many purposes. The sugar may be purified by recrystallisation from boiling 87-90 per cent. ethyl-alcohol (solubility about 1 in 20).

The hydrolysis of starch to dextrins and maltose is due in this case to the enzyme diastase present in the malt.

The preparation of lactose will be described in the chapter on milk.

VI. Preparation of an Amino-hexose,—Glucosamine or Chitosamine Hydrochloride.



Lobster or crab shells are freed as far as possible from the soft parts, decalcified by maceration with cold, dilute hydrochloric acid for two days, thoroughly washed with water and cut into small pieces, any still adherent muscle being removed. 50 grms. of this crude chitin are heated to the boiling-point in a round-bottomed flask fitted with a reflux condenser with about 200 c.c. of concentrated hydrochloric acid, gently boiled for about an hour, and then heated on the water bath for about three hours. Part of the hydrochloric

acid is then distilled off under reduced pressure. When a considerable amount of crystalline material has separated out, the mixture is diluted with two or three volumes of water, heated on the water bath, and again concentrated by distillation in vacuo until abundant crystallisation sets in. The crystalline mass is then dissolved in about five times its volume of water, boiled with animal charcoal for some time, and filtered. The filtrate is then concentrated in vacuo on the water bath until a considerable amount of glucosamine hydrochloride has separated out. The flask is then detached from the condenser, cooled thoroughly under the tap, stoppered, and left overnight in the ice chest. The crystalline precipitate is then filtered off with the aid of suction on a filter-paper placed in a Buchner funnel. The glucosamine hydrochloride may be purified by again dissolving it in warm water, adding animal charcoal, boiling, and again filtering. Colourless crystals of glucosamine hydrochloride separate out on concentrating the filtrate by distilling off the water in vacuo. The crystals are filtered off through a Buchner funnel, partially dried by suction with the water pump, and finally in vacuo over anhydrous calcium chloride.

Some reactions of chitosamine or glucosamine hydrochloride.

- (a) The crystals are faintly sweet, and also slightly saline.
- (b) Dissolve a small quantity in as little warm water as possible in a test-tube. Place a drop of the solution on a microscopic slide. If crystals do not separate out from the drop on standing for some minutes, concentrate the solution by evaporating very gently over a Bunsen burner, the slide being held some distance from the flame. On standing, large, somewhat thick, glancing plate-like crystals, frequently of roughly hexagonal shape, separate out. These have been found to belong to the monoclinic system.
- (c) Dissolve a small quantity of the crystals in a few c.c. of water, add about half the volume of a solution of caustic soda and heat. Note that ammonia is given off, and that the solution becomes yellow and then brown in colour (Moore's test).
- (d) Render another portion of the solution alkaline with caustic soda, and add a few drops of copper sulphate solution. A dark blue solution is obtained, and reduction occurs on heating.
- (e) Render another portion of the solution slightly acid with dilute nitric acid, and add a little silver nitrate, a precipitate of silver chloride is obtained. If the percentage of hydrochloric acid in a weighed quantity of the salt be determined by Mohr's method, a result will be obtained in close agreement with the formula already given.
- (f) A solution of chitosamine hydrochloride will be found to yield the same osazone on treatment with phenylhydrazine acetate as glucose; but the yield is small.
- (g) It is optically active ($[\alpha]_D = +73.7$ for a 10 per cent. solution).
- (h) It does not ferment with yeast.

Chitosamine or glucosamine combines in alkaline solution with phenylisocyanate to form a sparingly soluble addition product. A

method of separating chitosamine from mixtures containing amino-acids has been based on this fact. It is converted on oxidation with dilute nitric acid into a stereoisomeride of saccharic acid known as norisosaccharic acid (with its anhydride), and by nitrous acid into a nitrogen free derivative known as chitose ($C_6H_{10}O_5$). Free glucosamine, which is much less stable than the hydrochloride, may be prepared from its salts by decomposing the latter with diethylamine or sodium methoxide.¹

Glucosamine, and possibly other stereoisomeric substances, are widely distributed in nature, being found as products of the acid hydrolysis of mucins and other glucoproteins, and of the chondrin obtained from cartilage.

VII. Preparation of the Enzymes. (1) Emulsin ; and (2) Invertase.

(1) Emulsin—

Rub up 50 grms. of sweet almonds in a mortar, adding a little pure sand to facilitate their disintegration. The powder is then macerated for about 2 hours with about 100 c.c. of 1 per cent. acetic acid, and the extract filtered. This crude extract is highly active and may be used without further treatment, but has the disadvantage of reducing Fehling owing to the presence of glucose. The enzyme may be freed from glucose by the following method:—Add about 150 c.c. of absolute alcohol to the extract, allow the precipitate which forms to settle, and filter it off with the aid of suction by means of the filter pump. After the alcohol has evaporated, extract the residue with about 25 c.c. of water, and filter the solution obtained. A more permanent dry preparation of the enzyme may be prepared by again precipitating it with alcohol, washing with alcohol and ether, and drying the residue in vacuo over anhydrous calcium chloride, or preferably over soda lime.

Emulsin is the specific enzyme for β -alkyl glucosides. It must, however, be remembered that emulsin is a mixture of enzymes.

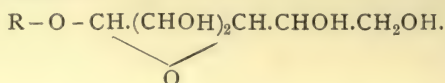
(2) Invertase—

Twenty grms. of yeast, previously washed with a little distilled water, are rubbed up with 200 c.c. of 95 per cent. alcohol, and the alcoholic suspension is left for about twelve hours in a stoppered vessel. The sediment is transferred to a Buchner funnel, filtered off, washed with alcohol and ether, partially dried by suction, and completely dried in an evacuated desiccator over anhydrous calcium chloride. The dried product, when kept in a well-stoppered bottle, retains its activity for a considerable time (at least nine months); 1 gm. of the dried product is extracted with 100 c.c. of water containing toluene. The filtered fluid is a highly active solution of invertase, which retains its activity for at least a week.

¹ See "The Simple Carbohydrates and Glucosides," by E. F. Armstrong, pp. 42-44, and p. 144.

VIII. Preparation and Properties of some Glucosides.

The glucosides may be defined as organic substances which yield on hydrolysis by enzymes or dilute acids one or more sugars and some other products. The other products vary greatly in their chemical structure, including alcohols, acids, phenols, etc. The glucosides correspond in structure to the simple methyl derivatives of glucose, and their general formula is consequently —



R represents the organic radicle other than sugar. 1. Amygdalin, 2. Salicin, and 3. Phloridzin, may be selected for investigation as types of the class.

1. **Amygdalin.**—($\text{C}_{20}\text{H}_{27}\text{NO}_{11}$. M.w. 457.4).

(1) PREPARATION.

Two hundred and fifty grms. of bitter almonds are dried for two hours in the hot water oven. The powdered mass is then extracted for several hours with petroleum ether in Soxhlet's extraction apparatus, to remove the fat. The crushed almonds, after being freed from petroleum ether by gentle heat, are extracted for about 15 minutes with about 300 c.c. of boiling 96 per cent. alcohol on the water bath in a flask fitted with a reflux condenser. The alcoholic solution is allowed to cool, and is then decanted and filtered. The solid residue may be extracted anew with 150 c.c. of fresh alcohol, and the alcoholic solution cooled and filtered. The united filtrates are then concentrated to a volume of about 100 c.c. by distilling off part of the alcohol under reduced pressure. The concentrated solution is then placed in a stoppered flask in the ice chest. Any crystals of amygdalin, which separate out, are filtered off and the filtrate is mixed with at least half its volume of ether. A further quantity of amygdalin, which separates out on standing, is also filtered off. The amygdalin is then dried in vacuo in the desiccator over anhydrous calcium chloride.

(2) PROPERTIES.

(a) Heat a small quantity of amygdalin in a dry test-tube. Anhydrous amygdalin melts simultaneously becoming yellow and then brown (m.p. about 200°), and gives off vapours having a distinct odour of bitter almonds. Amygdalin when crystallised from water contains three molecules of water of crystallisation, which are lost when the glucoside is heated to 110° - 120° .

(b) Its taste is very faintly bitter.

(c) Dissolve a small quantity in a few drops of water (solubility 8 in 100). The aqueous solution is neutral, and odourless. Place a

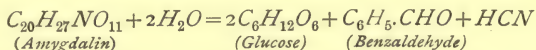
drop or two of the solution on a microscopic slide, and concentrate the solution by gently heating the slide over a Bunsen flame. On cooling, the amygdalin crystallises out as long rhombic prisms, frequently of considerable size.

(d) Dissolve another portion in warm 95 per cent. alcohol. Cool the alcoholic solution and divide into two equal portions. Add to one about an equal volume of ether, and shake vigorously. A crystalline precipitate separates out. On microscopic examination this is found to consist of spherical masses of fine needles. Concentrate the other half of the alcoholic solution by evaporation; long rhombic needle-shaped crystals separate out. Amygdalin is therefore readily soluble in alcohol and water, but insoluble in ether.

(e) Its specific rotatory power ($[\alpha]_D = -35.5$) may be determined if sufficient material is available.

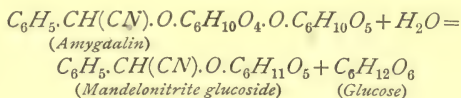
(f) Dissolve 0.2 grm. of amygdalin in 20 c.c. of water. Add to a small quantity of this solution about one-fourth its volume of caustic soda, and drop by drop copper sulphate. The cupric hydrate precipitate dissolves to form a clear blue solution, but no reduction occurs on heating.

(g) Action of dilute acids.—Add 6 drops of concentrated hydrochloric acid to 5 c.c. of the solution, and heat on the water bath for an hour. Cool the solution, render alkaline with caustic soda, add a few drops of copper sulphate solution, and heat. Reduction occurs owing to the presence of glucose resulting from the hydrolysis of the amygdalin. While heating, the odour of bitter almonds becomes perceptible.



(h) Action of emulsin.—Add 10-15 drops of the solution of emulsin to 10 c.c. of the aqueous solution of amygdalin and leave on the water bath at 40° to 45° for half an hour. Note the distinct odour of bitter almonds due to the presence of benzaldehyde and hydrocyanic acid. Carry out Trommer's test. Reduction occurs. The amygdalin has been hydrolysed by the enzyme yielding the same products as are formed by hydrolysis with dilute hydrochloric acid.

When amygdalin is acted on by an enzyme, which can be extracted with water from previously washed and dried yeast, only one molecule of glucose is split off, thus:—



It is noteworthy that yeast which has been macerated with alcohol does not yield this enzyme, which is therefore absent from solutions of invertase prepared by the method already described. Further experimental evidence has proved that emulsin really consists of two enzymes, one of which, known as amygdalase, hydrolyses amygdalin in the same

way as the yeast enzyme, while the other, known as prunase, splits off the second molecule of glucose (Armstrong, *l.c.*, pp. 116-120).

2. Salicin ($C_{13}H_{18}O_7$, M.w.286).

(1) PREPARATION.—Salicin may be obtained by extracting willow bark (*e.g.* bark of *Salix helix*) with boiling water, filtering the extract and digesting it with lead oxide, to remove colouring matters and extractives. The extract is again filtered freed from lead with sodium sulphate, filtered, and concentrated by evaporation. The glucoside separates out on standing.

(2) PROPERTIES—

(a) Heat a small quantity in a dry test-tube. The glucoside melts to form a colourless fluid, which rapidly becomes brown and chars on further heating (m.p. 201°).

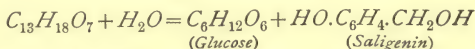
(b) Dissolve a small quantity in cold concentrated sulphuric acid. An intensely red solution is obtained.

(c) Dissolve a small quantity in warm water, examine microscopically the crystals, which separate out on cooling (solubility in cold water, 3 in 100). If the solution is not sufficiently concentrated, evaporate a few drops on a slide. The crystals are colourless prismatic needles. Note that the aqueous solution is neutral in reaction and has an intensely bitter taste.

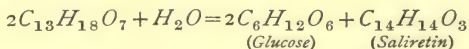
(d) Render a portion of the aqueous solution alkaline with caustic soda, and add a few drops of copper sulphate. Little cupric hydrate is dissolved, and no reduction occurs on heating.

(e) Add a few drops of dilute ferric chloride to another portion of the solution, no colour change occurs.

(f) Add a little emulsin (about 15 drops) to 10 c.c. of 1 per cent. solution of salicin, and leave on the water bath at 40° to 45° for half an hour. Divide the fluid into two portions. Test one by Trommer's method. Distinct reduction occurs owing to the presence of glucose formed by the hydrolytic action of the enzyme on the glucoside. Add a few drops of dilute ferric chloride to the other portion. A deep violet-blue colour is obtained due to the formation of a coloured compound of saligenin with ferric chloride. The salicin has been hydrolysed by emulsin, thus:—



(g) Add 5 drops of concentrated hydrochloric acid to 5 c.c. of 1 per cent. solution of salicin, and heat in the water bath for fifteen minutes. Render the solution alkaline and test with Trommer's or Fehling's solution, reduction occurs. The salicin has been hydrolysed by the dilute acid, thus:—



Saliretin is probably an anhydride of saligenin.

(h) The solutions of salicin are *lævo-rotatory*. $[\alpha]_D = -64.90$.

3. Phloridzin ($C_{21}H_{24}O_{10} + 2H_2O$, M.w.472.3) is of great physio-

logical interest on account of its well-known property of producing glycosuria.

(1) PREPARATION.—Phloridzin may be obtained by extracting the cortex of the roots of the apple tree with boiling dilute alcohol. The glucoside crystallises out from the alcoholic extract after concentration by distilling off part of the alcohol. It may be purified by recrystallisation from warm water with the addition of animal charcoal.

(2) PROPERTIES.

(a) Heat a small quantity of the anhydrous glucoside in a test-tube. It melts to form a clear, colourless fluid, which rapidly darkens on further heating and ultimately chars (m.p. of anhydrous glucoside = 170° m.p. of glucoside + $2H_2O = 108^{\circ} - 109^{\circ}$).

(b) Dissolve a small quantity of phloridzin in warm water. On cooling, phloridzin separates as fine, colourless needles. It is sparingly soluble in cold water (1 in 1000), very readily soluble in warm water. It also dissolves readily in alcohol and acetone, but is practically insoluble in ether. Its aqueous solutions are neutral in reaction, and only very faintly bitter.

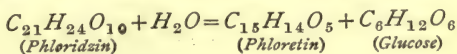
(c) Phloridzin readily dissolves in cold dilute aqueous solutions of the caustic alkalies, of sodium and potassium carbonate, and of ammonia. The alkaline solutions absorb oxygen and undergo decomposition, becoming red-brown in colour.

(d) Aqueous solutions of phloridzin give a dark reddish-violet colour with ferric chloride. They do not reduce either Fehling or Benedict's (qualitative) reagents.

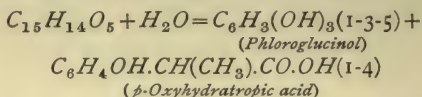
(e) Hydrolysis of phloridzin.

(a) Acid hydrolysis.—Dissolve 0.1 grm. of phloridzin in 10 c.c. of warm water, add four drops of concentrated hydrochloric acid, heat to the boiling-point, and place on the water bath. The solution, which is at first clear, commences to become turbid in about fifteen minutes. This turbidity is due to the separation of phloretin, which, unlike phloridzin, is sparingly soluble in boiling as well as in cold water. When the fluid has remained at least forty minutes on the water bath, it is cooled and filtered. Portions of the filtrate are rendered alkaline with sodium carbonate, and tested with Fehling's and Benedict's reagents. Reduction occurs in both cases owing to the presence of glucose which may be identified by other reactions.

The crystalline precipitate of phloretin (m.p. 180°) is washed with a little cold water. It is a neutral, almost tasteless (very faintly sweet) substance, and its alcoholic solution is optically inactive, while phloridzin is lævo-rotatory. It is readily soluble in alcohol, from which it may be crystallised as long colourless plates, is almost insoluble in cold and warm water, and very sparingly soluble in ether. It dissolves readily in alkalies, yielding solutions which absorb oxygen from the air, and become yellow, and then red-brown. The following equation represents the course of the hydrolysis:—



When boiled with caustic potash, phloretin is decomposed, yielding phloroglucinol and *p*-oxyhydratropic acid, thus:—



(β) Phloridzin is not hydrolysed by emulsin. Add 15 drops of emulsin to 0.1 gm. of phloridzin suspended in 10 c.c. of water, and leave on the water bath at 40° to 45° for an hour. Test portions of the fluid with Fehling's or Benedict's reagents. No reduction occurs if the solution of emulsin be free from glucose.

IX. Preparation and Properties of some Polysaccharides.

1. **Starch** $[(C_6H_{10}O_5)_n]$ may readily be obtained from potatoes and cereals by a simple process, the outlines of which will be given in Chapter V. Sufficiently pure starch is prepared industrially on a large scale from potatoes and cereals, and is therefore so readily procurable that its separation from natural products is rarely carried out in the laboratory.

2. **Glycogen** $[(C_6H_{10}O_5)_n]$. Brücke's method of preparing glycogen is described in Chapter XI. The following method (Fraenkel) is a very rapid one and yields a fairly pure product.

One part of fresh liver excised from a rabbit, previously fed on a diet rich in carbohydrates, is rubbed up in a mortar with three parts of 3 per cent. trichloroacetic acid. The extract is then filtered with the aid of suction by the filter pump through filter-paper placed in a Buchner funnel. The glycogen is precipitated from the opalescent filtrate by the addition of alcohol, the precipitate is filtered off, washed with alcohol and ether, and dried in vacuo over anhydrous calcium chloride.

3. **Erythrodextrin** $[(C_6H_{10}O_5)_n]$. Dextrins, mainly achroödextrins, were obtained as a by-product in the preparation of maltose. Erythrodextrin may be prepared by the following method:—

Twenty c.c. of 9 per cent. solution of oxalic acid are heated in a flask on the water bath. 10 grms. of potato starch are rubbed up in a mortar with 20 c.c. of cold water. The starch paste is then gradually added to the oxalic acid solution, which is heated on the water bath for about 15 minutes. Any starch adhering to the sides of the flask is finally washed down with about 20 c.c. of warm water. The mixture is frequently shaken, kept on the water bath for half an hour, then transferred to a porcelain basin and heated for about another half-hour, until a drop of the solution gives a pure red-brown colour with iodine. The concentrated solution is then cooled, measured, and mixed with about five times its bulk of methylated

spirit. The suspension of dextrin is then allowed to stand for 12 hours, the supernatant fluid is decanted, the precipitate of dextrin transferred to filter-paper, washed with 80 per cent. then 95 per cent. alcohol, and dried in vacuo over anhydrous calcium chloride.

4. Inulin [$(C_6H_{10}O_5)_n$]. (I) PREPARATION.

250 grms. of crushed *Dahlia* tubers, collected in October, are mixed with about 15 grms. of calcium carbonate to neutralise organic acids, and heated on the water bath with 600 c.c. of water for about an hour. The warm solution is filtered through well-washed calico, concentrated to about 150 c.c., then mixed with an equal volume of 95 per cent. alcohol, filtered after standing for about 15 minutes, and the filtrate is left for at least 24 hours in the ice chest.

The impure inulin, which separates out, is filtered off with the aid of suction by the filter pump, washed with 70 per cent. alcohol, and dissolved in as small a quantity of warm water as possible. The aqueous solution is cooled and left for about 48 hours in the ice chest. Inulin gradually separates out as a white deposit, which is filtered off and dried in vacuo. It may be purified by again dissolving it in warm water and allowing it to separate out on cooling. The final product appears to be a mixture of polysaccharides, which all yield fructose on hydrolysis with dilute acids.

Inulin may also be prepared from *radix inulæ*, or from *radix pyrethri*, which can be obtained as powders from any firm of pharmaceutical chemists. It may also be prepared from artichokes.

(2) PROPERTIES OF INULIN.

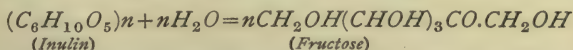
(a) Dissolve 0.2 gm. of inulin in 20 c.c. of warm water. It readily dissolves, yielding a transparent solution. It is only very sparingly soluble in cold water (1 in 10,000 according to Tanret), but, when heated with water, readily yields supersaturated solutions from which it only separates out slowly on cooling. The method of preparation is largely based on these properties.

(b) It is insoluble in alcohol and ether, and may be precipitated from its aqueous solution by the addition of excess of alcohol.

(c) Place 5 c.c. of the aqueous solution of inulin in a test-tube, add about one-fourth its volume of caustic soda solution, and a few drops of copper sulphate solution. The cupric hydrate is not dissolved, and no reduction occurs on heating.

(d) Add a few drops of iodine dissolved in a solution of potassium iodide to a small quantity of the aqueous solution of inulin, and, as a control, the same amount of iodine to a small quantity of water. No colour change occurs.

(e) Add two or three drops of concentrated hydrochloric acid to 5 c.c. of the solution of inulin, boil for one or two minutes, neutralise, and test with Fehling's reagent. Reduction occurs, owing to the inulin having been hydrolysed to fructose.



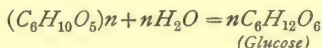
(f) Add a few granules of resorcinol to a small quantity of the

solution of inulin and about one-fourth the volume of the solution of concentrated hydrochloric acid. On heating the solution on the water bath, a dark red-coloured solution is obtained, from which a red precipitate, soluble in alcohol (ethyl or amyl), gradually separates out.

5. Cellulose $[(C_6H_{10}O_5)_n]$. Ash free filter-paper, which consists mainly of cellulose, may be used for the tests.

Cellulose is insoluble in most solvents. It dissolves in ammoniacal solutions of cupric hydrate, and after treatment with alkali, in carbon bisulphide.

(1) Hydrolysis of cellulose.—Mix in a small flask 15 c.c. of concentrated sulphuric acid with about 9 c.c. of water, allow the mixture to cool completely, then add gradually 4 to 5 grms. of finely cut filter-paper, shaking thoroughly after each addition. When a more or less homogeneous mixture has been obtained add the contents of the flask to 400 or 500 c.c. of water, and boil this solution for an hour and a half. Neutralise a portion with 30 per cent. caustic alkali, and test with Trommer or Fehling's solutions. Reduction occurs, the cellulose having been converted into glucose.



(2) Colour reaction with iodine.—Place two pieces of filter-paper in separate porcelain basins. Add to one a drop of an aqueous solution of iodine, and on the same portion of the filter-paper a drop of a thoroughly cooled mixture of 2 vols. of sulphuric acid with 1 vol. of water. Place a drop of the sulphuric acid on the other filter-paper, which serves as a control. The part of the first filter-paper which has been treated with iodine and sulphuric acid becomes dark blue. The cellulose has been converted by the action of the acid into an amyloextrin, which gives a blue colour with iodine. The filter-paper sometimes gives a faint blue colour reaction with iodine alone; but this is greatly intensified by the action of the sulphuric. If the sulphuric acid solution be thoroughly cool, no charring of the second filter-paper ought to occur.

CHAPTER III

FATS AND SOME ALLIED SUBSTANCES

INTRODUCTION

THE next group of nitrogen-free organic substances is that of the Fats.

Animal fats consist mainly of the neutral esters of glycerol $[C_3H_5(OH)_3]$ with palmitic $(C_{15}H_{31}CO.OH$, melting point $+62.6^\circ$, boiling point at 100 mm. of Hg $+268^\circ$), stearic $(C_{17}H_{35}CO.OH$, M.p. $+69.3$, B.p. at 100 mm. $+287^\circ$), and oleic $[CH_3.(CH_2)_7.CH:CH.(CH_2)_7.CO.OH$, M.p. $+14^\circ$, B.p. at 10 mm. $+223^\circ]$ acids. These glycerides are known as tristearin $[(C_{17}H_{35}CO.O)_3C_3H_5$, melts at $+55^\circ$, solidifies on further heating, and melts again at $72^\circ]$, tripalmitin $[(C_{15}H_{31}CO.O)_3C_3H_5$, M.p. $+61^\circ]$, and triolein $[(C_{17}H_{33}CO.O)_3C_3H_5$, M.p. $-5^\circ]$.

All the neutral fats or glycerides are decomposed by heat yielding acrolein $(CH_2:CH.CHO)$ and other products. They are all of lower specific gravity than water, and when pure are colourless, odourless, and of neutral reaction. On exposure to the air they gradually become yellow, and acquire an unpleasant odour and taste, and acid reaction—in other words, they become “rancid.” These changes, which are largely due to the action of bacteria, consist in the partial hydrolysis of the fats to glycerol and free fatty acids, followed by oxidation of the latter to volatile substances having an unpleasant odour.

The consistence and melting points of the natural fats vary according to the relative proportions of the solid and liquid

glycerides which are present. Those in which olein preponderates are liquid at ordinary temperatures (15° to 25°), and are known as oils. Mixed glycerides containing two or three different fatty acid radicles, *e.g.* dioleostearin, are also present in natural fats. Some fats, especially butter, also contain glycerides of lower fatty acids, such as butyric ($C_4H_8O_2$), caproic ($C_6H_{12}O_2$), caprylic ($C_8H_{16}O_2$), and capric ($C_{10}H_{20}O_2$). All the fatty acids mentioned, with the exception of oleic, are saturated, all contain an even number of carbon atoms and, with that exception, have the general formula ($C_nH_{2n}O_2$).

The neutral fats are insoluble in water, and in aqueous solutions of the caustic alkalies, so long as saponification has not occurred, sparingly soluble in cold, more readily soluble in hot, ethyl alcohol; sparingly soluble in acetone; readily soluble in ether, ligroin, benzene, chloroform, and carbon tetrachloride. These solvents are frequently used in extracting fats from dried animal tissues. Most fats also contain traces of lecithin, and cholesterol or other sterols.

A number of complex substances termed lipins (Leathes), which are closely related to the simple fats, have also been isolated from the tissues. Nerve tissue is particularly rich in these. They resemble the simple fats in yielding fatty acids, or hydroxy-fatty acids on hydrolysis; but differ from them in also containing either nitrogen or nitrogen and phosphorus. The isolation of the lipins presents great technical difficulties, and the elucidation of their chemical composition and structure is still far from complete. They have been divided into two main groups—(1) the phospholipins or phosphatides, containing phosphorus and nitrogen, and (2) the galacto-lipins (glyco-lipins or cerebrosides), which are free from phosphorus.

The following table, which is necessarily of a provisional character, contains a list of the chief lipins, with the products of their hydrolysis:—

	$\frac{N}{P}$	Products of hydrolysis.
I. Phospho-lipins . .		Various fatty acids (saturated and unsaturated) and phosphoric acid are yielded by all.
(1) Monamino-mono-phospho-lipins	$\frac{1}{1}$	Additional products of hydrolysis.
(a) Lecithin .		Glycerol and choline $[\text{HO} \cdot (\text{CH}_2)_2 \cdot \text{N}(\text{CH}_3)_3 \cdot \text{OH}]$.
(b) Cephalin .		Glycerol, amino - ethyl alcohol $(\text{CH}_2\text{OH} \cdot \text{CH}_2\text{NH}_2)$, NH_3 , and probably other amine derivatives.
(2) Diamino - mono-phospho-lipin, (a) Sphingomyelin	$\frac{2}{1}$	An alcohol (sphingol?), two bases sphingosine $[\text{CH}_3 \cdot (\text{CH}_2)_{11} \cdot \text{CH} : \text{CH} (\text{CHOH})_2 \cdot \text{CH}_2\text{NH}_2]$, and choline.
(3) Monamino-diphospho-lipin (a) Cuorin (?)	$\frac{1}{2}$	Glycerol and an unknown base.
II. Galacto-lipins or cerebrosides		Products of hydrolysis, galactose, ¹ sphingosine, and phrenosinic acid $[\text{CH}_3 \cdot (\text{CH}_2)_{22} \cdot \text{CHOH} \cdot \text{CO} \cdot \text{OH}]$ and
(a) Phrenosin		
(b) Kerasin		lignoceric acid $[\text{CH}_3 \cdot (\text{CH}_2)_{22} \cdot \text{CO} \cdot \text{OH}]$.

Solutions of all the lipins given in this table are dextrorotatory with the exception of kerasin, which is lævorotatory. The optical properties of cuorin have not been determined. The methods of isolation and properties of lecithin, which may be regarded as a type of the phospho-lipins, are described in Chapter V., page 92.

The partial separation of some lipins from nerve tissue will be described later. The first step in their separation is usually based on the fact that the lipins are practically insoluble in cold acetone, while

¹ The first two products are common to both cerebrosides.

cholesterol, neutral fats, and fatty acids dissolve more or less readily in in that solvent.¹

The following tests may be carried out with such a mixture of fats as occurs in beef- or mutton-fat, or in an oil such as palm- or castor-oil.

If the fat be a solid one, at room temperature, then it is advisable to take the melting point first of all.

[(a) Take a small quantity of fat in a very short test-tube of small calibre, or, best of all, in a small piece of glass tubing with one end fused. With a rubber band fix the tubing just above the bulb of a thermometer, and hold the latter in a beaker of water at a temperature of about 15°. Place in the beaker a suitable glass stirrer, the circular end of which surrounds the tube and thermometer. Gradually warm the water, stirring it so as to allow of equal distribution of heat, and note the temperature at which the fats melt. The natural fats being mixtures and not definite chemical substances do not possess well-defined melting points, and the results obtained by different observers are consequently somewhat discordant. The determination of the melting points of the fats is consequently difficult and uncertain.]

(b) Pour a little of the melted fat upon a piece of paper.

Note the grease spot from the melted fat penetrating the paper. The grease spot remains on heating the paper, while the similar spot produced by a volatile oil, *e.g.* clove oil, is removed more or less completely by heat.

(c) Add a drop of phenolphthalein to a few c.c. alcohol in a test-tube, and one or two drops of very dilute alkali, just sufficient to give the solution a red colour. Now add, drop by drop, an ethereal solution of the melted fat, and

¹ The reader is referred to the monographs on "The Fats," by J. B. Leathes, and on "Lecithin and Allied Substances," by H. Maclean, for full details, and references to the literature of this subject.

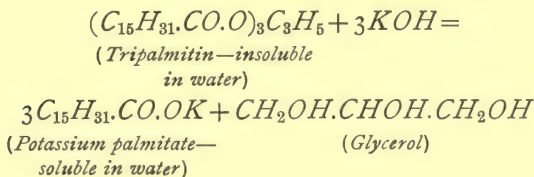
shake; if the red colour does not disappear, then the fat mixture does not contain free fatty acids.

The ether must of course be neutral in reaction.

(d) SAPONIFICATION.

On one water bath in the laboratory keep a fairly large quantity of the fat melted in a porcelain basin, and on another keep a flask (fitted with upright condenser) containing a strong alcoholic solution of potash, at a temperature slightly below boiling point.¹ Pour some of the latter into a flask kept in hot water bath, and immediately pour in some of the melted fat. Mix thoroughly and keep on hot water bath until saponification is complete (takes place rapidly). Occasionally take out a small quantity and shake up gently in a little distilled water. If saponification be finished, no oil drops will rise to the top.

The following equation expresses the chemical reaction which occurs in the case of tripalmitin. The tristearin and trolein also present undergo a similar decomposition:—

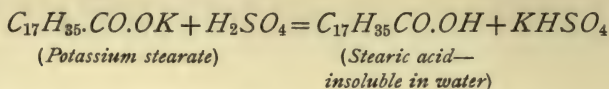


(e) DECOMPOSITION OF THE SOAP.

Warm some 20 per cent. H_2SO_4 in a flask, and gradually add some of the soap solution to it; the fatty acids separate out as an oily layer on the top. Cool, filter, and wash the fatty acids with distilled water until free from the H_2SO_4 . With this deposit carry out the tests for fatty acids (f), (g), and (h).

¹ Dissolve 30 grms. KOH in 20 c.c. water in water bath, and then pour this into 200 c.c. 90 per cent. alcohol kept in a litre flask.

The equation expressing the chemical reaction, which occurs, may be written:—



The potassium palmitate and oleate also present are decomposed in the same way by the action of the sulphuric acid, palmitic and oleic acids being set free.

(f) Dissolve some fatty acid in ether, and add to slightly alkaline phenolphthalein; the red colour is discharged.

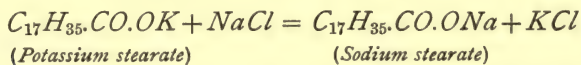
(g) Dissolve some in NaOH, and shake up with warm water. Soap lather is produced.

Keep this for Tests (i) and (j).

[(h) Heat some of the fatty acid in dry test-tube with acid potassium sulphate. No smell of acrolein (if the fatty acid is free from glycerol).]

(i) Add a small quantity of cod-liver oil or olive oil to some soap solution in a test-tube. An emulsion is formed on shaking. As a control, shake up a few drops of the oil with water.

(j) Heat a small quantity of the soap solution prepared as described in (g), and saturate it with finely powdered sodium chloride, a precipitate of sodium soaps separates or is "salted out." If a similar experiment be carried out with a portion of the solution of potassium soaps obtained in (d), the potassium soaps are first converted into sodium soaps and then "salted out."



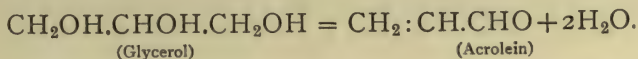
The potassium soaps, commonly known as "soft soaps," are more soluble in water than the corresponding sodium soaps, known as "hard soaps."

(k) The presence of glycerol in the fat may be detected by testing either the syrupy residue left on evaporation of

filtrate from the fatty acids, or a small quantity of the fat itself. Mix up thoroughly some fat or one drop of glycerol with KHSO_4 in a dry mortar, then transfer to dry test-tube and heat carefully.

The pungent odour of acrolein can then easily be detected at some distance off.

The following equation represents the chemical change which occurs:—



(l) The presence of lower fatty acids can be detected on smelling the warm acid fluid obtained during the decomposition of the soaps.

The amount of glycerides of volatile fatty acids present in most animal fats, such as lard, is small and the rancid odour due to the presence of volatile fatty acids is therefore not distinct. On the other hand, butter fat yields about 7 per cent. of volatile fatty acids having a characteristic rancid odour.

[(m) The melting point of purified fatty acids can be taken and compared with that of the original fat. The temperature of solidification, or solidifying point, is more sharply defined, and consequently more easily measurable.]

An adequate description of the chemical methods of analysing the fats is beyond the scope of this book. The reader is referred to "Practical Organic and Bio-Chemistry," by R. H. A. Plimmer (pp. 179-182), for practical details. Both the physical and chemical methods of examination are given in "The Chemical Technology and Analysis of Oils, Fats, and Waxes," by J. Lewkowitsch, vol. i. pp. 224-403.

CHAPTER IV

PROTEINS

INTRODUCTION

General Characters of the Proteins.

WE now come to the most important class of organic substances with which we have to deal, namely, the proteins. All of these contain carbon (about 52 per cent.), nitrogen (about 16 per cent.), hydrogen (7 per cent.), oxygen (22 per cent.), practically all sulphur (0.5 to 2.3 per cent.), many phosphorus, some iron or copper, and a few iodine. The amounts of the elements present vary considerably in different proteins, and the foregoing figures are therefore only approximate.

The proteins are, as a rule, practically tasteless and odourless substances, which usually separate out in an amorphous form when obtained in the solid state, either by concentration of their aqueous solutions at a low temperature or by precipitation with neutral salts. Many have also been obtained in the crystalline form.

Diffusibility, Osmotic Pressure, and Related Properties.

Proteins have an extremely slow rate of diffusion through aqueous media, and are practically unable to pass through solid colloidal membranes, such as parchment, paper, gelatin, or collodion membranes. The term "colloid" was applied to substances having these properties by Graham in 1861; while he designated as "crystalloids" all substances—both non-electrolytes, such as sugar and urea and electrolytes—which

diffuse rapidly through aqueous media, and are not arrested by colloidal septa. The most essential difference between colloids and crystalloids is that the colloid particles in suspension, whether molecules or aggregates of molecules, are relatively large; while the crystalloid particles, whether molecules or ions, are relatively small. Most protein solutions or sols are "reversible" (Hardy). If the solid protein, whether precipitated by the addition of neutral salt to its solution, or obtained as a residue after evaporation, will dissolve again in water, the solution is said to be reversible. Reversible colloids are also frequently known as emulsoids.¹

The fact that solid colloidal membranes are readily permeable to crystalloids but not to proteins or other colloids, has been applied to the separation of colloids from crystalloids by the processes known as dialysis and ultra-filtration, which will be studied experimentally at a later stage.

Solid colloidal membranes have also been used as semi-permeable membranes in the construction of instruments known as osmometers, for determining the osmotic pressures of solutions of proteins and other colloids, the influence of temporary variations in osmotic pressure due to crystalloids being usually eliminated by allowing the apparatus to stand until the concentrations of crystalloids on the two surfaces of the membrane have been equalised by diffusion. The residual persistent osmotic pressure due to the dissolved colloid is then measured by a manometer connected with the chamber containing the colloid solution. The osmotic pressure exerted by the proteins of blood serum has been found to amount to 25-30 mm. of mercury (Starling). A 10 per cent. solution of gelatin yields a persistent pressure of 70 mm. of mercury at 26° (Moore and Roaf). The osmotic pressure of colloids increases with rising temperature more rapidly than if it were proportional to the absolute temperature. As a rule the osmotic pressure of proteins is lowered in the presence of neutral salts. This reduction in pressure is to be regarded as due to the coalescence of protein particles to form a smaller number

¹ The reader is referred to text-books on the colloids for a description of the properties of irreversible or suspensoid colloids.

of larger aggregates. This process of aggregation, resulting from low concentrations of neutral salts, may be interpreted as the first step towards the precipitation produced by the addition of larger quantities of neutral salts up to the point of saturation.

When the osmotic pressure of a solution has been determined, the molecular weight of the solute may be calculated on the assumption that the osmotic pressure is directly proportional to molar concentration and absolute temperature. Although the validity of the latter assumption has been seen to be doubtful for colloidal solutions, Hüfner and others have calculated the molecular weight of hæmoglobin from their osmotic pressure results, and obtained a value of 15,000 to 16,000, which agrees well with the value deduced from chemical data.

The depressions of the freezing point, caused by protein solutions, when freed as far as possible from crystalloids by dialysis, are so slight that molecular weight determinations based on such results have little value. The latter conclusion may be justified by considering the relationship between osmotic pressure and depression of freezing point. If P represents the osmotic pressure in atmospheres of a solute dissolved in water, and T the lowering of the freezing point, it can be proved that $P = 12.03 T$. Thus a depression of $.001^\circ$ is equivalent to an osmotic pressure of .012 atmospheres, or about 9.12 mm. of mercury. The latter is an easily measurable quantity; while the former value lies within the limits of experimental error.

Viscosity, Surface Tension, and related Properties.

The internal friction or viscosity of water is raised, and its surface tension lowered by protein solutes. In consequence of these properties protein solutions or sols are liable to undergo certain changes. Willard Gibbs and others have proved that solutes, which reduce the surface tension of a solvent, become more concentrated in the surface layer than throughout the bulk of the fluid. This process of surface concentration readily leads in the case of protein sols to the

formation of surface membranes having so high a degree of viscosity that they offer considerable resistance to distortion by mechanical forces. The formation of membranes is greatly accelerated by increasing the surface area either by mechanical shaking or by passing bubbles of air through the solution. Albumin has thus been almost completely separated from solution by mechanical shaking (Ramsden). This separation is to a large extent an irreversible process. The characteristic persistence of froth when formed on the surface of protein solutions is to be explained in a similar way. The protein membranes already described are formed at the interface or boundary surface between air and an aqueous protein solution. Similar membranes may be formed at the interface between immiscible or partially miscible liquids, and at the interfaces between solid particles and fluids. The protein membranes enclosing the fat globules of milk are formed in this way at the interfaces between the oil globules and the aqueous solution of milk proteins. The term "adsorption" has been applied to such interfacial changes in concentration.

ELECTRICAL PROPERTIES OF PROTEIN SOLS.

Conductivity.—Protein sols, when freed from crystalloids by dialysis, are practically non-conductors.

Migration of Particles in an Electric Field.—Some protein particles are found not to migrate in a definite direction under an electric field, others migrate towards the cathode, and others towards the anode. The protein particles belonging to the first class are uncharged or "isoelectric" (Hardy); those of the second class are positively charged (protein-cations); while those of the third class are negatively charged (protein-anions); albumin, hæmoglobin, and gelatin belong to the first class, histones probably belong to the second class; while nucleoproteins, phosphoproteins, glycoproteins, and most globulins belong to the third class. On the addition of dilute acids the protein particles of the first group acquire a positive charge, and migrate towards the cathode, while, on addition of dilute alkali, they acquire a negative charge and migrate towards the anode. Their sols

are stable at the isoelectric point, as well as in faintly acid or alkaline solution.

If dilute acid be gradually added to sols of the third class, they lose their charge at a certain reaction or hydrion concentration known as the "isoelectric point," and are precipitated. Their sols are therefore unstable at the isoelectric point. The hydrion concentration at the isoelectric point varies in different protein sols of the third class, and is characteristic for each protein. On the further addition of acid, the protein precipitate dissolves more or less completely, and the protein particles are now found to have acquired a positive charge. The behaviour of the histones does not appear to have been fully investigated. The experimental determination of the hydrion concentration at the isoelectric point for proteins of the third group is a comparatively simple process, which will be studied practically at a later stage, while the practical study of the migration of particles in an electric field lies outside the scope of the present book. The isoelectric point has been found to be the point of optimal precipitability, optimal agglutination, minimal solubility, and minimal viscosity (Michaelis). The Brownian movement also ceases at this point. Its determination is therefore of great practical importance.

Oppositely charged colloids mutually precipitate one another. Thus histone solutions yield precipitates with casein, serum globulin, and less readily with egg albumin. Further, a suspensoid, such as the sol of ferric hydrate, the particles of which are positively charged, precipitates most proteins, especially those carrying a negative charge. In faintly acid solution the proteins are precipitated by many suspensions, such as those of kaolin and mastix. Methods for freeing solutions from proteins have been based on these results.

Precipitation of Proteins.—The methods of precipitating proteins are dealt with in subsequent pages. For the separation of certain proteins as gels, and the properties and structure of the latter, the reader is referred to the literature given at the close of this introductory sketch.

Optical Properties.—All the simple proteins are lævoro-rotatory, but many of the compound proteins, such as the nucleoproteins and hæmoglobin are dextrorotatory. The rotations vary considerably according to the reaction of the solution.

The demonstration of the heterogeneity of protein sols by means of the "Tyndall effect," and with the ultra-microscope will be found described in text-books on the colloids.¹ The latter method has proved less effective for protein sols than for many suspensoids.

PROTECTIVE ACTION OF PROTEIN SOLUTIONS.—Suspensoid or irreversible colloidal sols differ from emulsoid or reversible ones in being readily precipitated by small quantities of electrolytes. This precipitation is inhibited to a greater or less degree by the addition of a solution of a reversible colloid, such as certain proteins.

GENERAL CHEMICAL CHARACTERS OF THE PROTEINS.

Molecular Weight.—The minimal molecular weight may be calculated from the percentage of any element present in small amount, such as sulphur or iron. Thus casein contains about 0.8 per cent. of sulphur, the atomic weight of sulphur is about 32, and since one molecule of casein (or caseinogen) must contain at least one atom of sulphur, the minimal molecular weight of casein would be $\frac{3200}{0.8}$ or 4000. Chemical evidence has proved that casein contains sulphur in more than one form of combination, and must, therefore, contain more than one atom of sulphur. The molecular weight of casein is, therefore, 4000 n , the value of n being uncertain but not less than 2.

Similarly oxyhæmoglobin (blood of horse) contains 0.335 per cent. of iron (atomic weight 55.84), its minimal molecular weight is, therefore, $\frac{5584}{0.335}$ or 16,669. This value agrees well

¹ See chapters v. and vi. of "The Physical Properties of Colloidal Solutions," by E. F. Burton, and chapter v. of "The Chemistry of the Colloids," by W. W. Taylor.

with those deduced from osmotic pressure measurements, and from the oxygen capacity of hæmoglobin.

Chemical Properties and Products of their Hydrolysis.—Proteins, when hydrolysed by acids, alkalies or enzymes, yield a large number of amino acids, of which eighteen have been isolated. The percentages and number of individual amino acids vary according to the nature of the protein examined. All these amino acids contain one or more amine, and one or more carboxyl groups, yet the following evidence indicates that the intact protein molecules possess few free amine or carboxyl groups. The protein molecule resembles the amino acids, into which it can be resolved, in having amphoteric characters. It can combine with acids to form salts in which the protein plays the part of a base or cation, this property being dependent on free amine groups. On the other hand, it can unite with bases to form salts in which the protein acts as an anion in consequence of the presence of free carboxyl groups. The combining power of protein both with acids and bases is small and varies according to the nature of the protein. The basic character predominates in the protamines, while the acid character preponderates in the nucleoproteins, phospho-, and glucoproteins. One protein molecule probably combines, as a rule, with not more than two to four molecules of a strong monobasic acid. The formaldehyde titration method, and Van Slyke's method, which will be described later, both yield results indicating that the free amine and carboxyl groups are few. Thus Van Slyke found that only 5-8 per cent. of the total protein nitrogen is set free by the action of nitrous acid. When protein is hydrolysed, the number of amine groups set free gradually increases, and there is a similar rise in the number of free carboxyl groups. From the fact that the majority of the amine and carboxyl groups of the intact protein molecule are not free, the conclusion may be drawn that they are probably united to one another. This inference is confirmed by the results of the synthesis of the polypeptides by E. Fischer. The view now held is that the majority of the amine groups

are united with the carboxyl groups as acid amides, thus:—



The organic radicles R and R' may be represented by hydrogen, or may belong to the aliphatic, isocyclic, or heterocyclic series.

The terminal free amine and carboxyl groups may remain free, unite together to form an internal salt, or form amide linkages with other amino acids. Such a chain of combined amino acids is known as a polypeptide, the number of amino-acid units when known being indicated by the prefix—di, tri, tetra, etc. Although the amide method of linkage is probably the chief one in proteins, other methods of linkage also occur, *e.g.* by means of sulphur, and the types of linkage found in arginine.¹

CLASSIFICATION OF THE PROTEINS proposed by the British Chemical and Physiological Societies, with certain modifications suggested by the American Physiological Society:—

I. Simple Proteins.

1. *Protamines*.—Strongly basic, yielding large quantities of diamino acids, soluble in ammonia, and not coagulable by heat. The majority give no protein colour reactions, except the biuret reaction.

2. *Histones*.—Strongly basic, soluble in water and dilute acids, insoluble in ammonia, and not completely coagulable by heat. Xanthoproteic, Millon's, and biuret reactions positive. Examples, histone (thymus) and globin.

3. *Albumins*.—Soluble in water, and dilute neutral salt solutions, and coagulable by heat. Serum albumin, ov-, and lactalbumins.

¹ The reader is referred to the following books for additional information, and for the literature of this subject:—"The General Characters of the Proteins," by S. B. Schryver; "Principles of General Physiology," by W. M. Bayliss (especially chapters iii. to v.). Publishers of both, Longmans, Green & Co. "Physical Chemistry," by J. C. Philip (chapters ix. to xi.). E. Arnold.

4. *Globulins*.—Insoluble in water, soluble in dilute neutral salt solutions, and coagulable by heat. Serum, and egg globulins, fibrinogen, and many others.

5. *Glutelins*.—Plant proteins. Insoluble in water and dilute neutral salt solution, soluble in dilute acids and alkalies, and coagulable by heat. Glutenin of wheat.

6. *Gliadins* (Prolamines).—Plant proteins. Insoluble in water, soluble in 70 to 80 per cent. alcohol. Gliadin, hordein, and zein.

7. *Scleroproteins* (Albuminoids).—Insoluble in water, neutral salt solutions, dilute acids or alkalies. Elastin, keratin, and collagen. The albuminoid gelatin is soluble in all the solvents mentioned, and is not coagulated by heat.¹

8. *Phosphoproteins* (sometimes included amongst the conjugated proteins).—Caseinogen and vitellin. For properties see later pages.

II. Conjugated Proteins.

These are compounds of a simple protein, with some additional or "prosthetic" substance.

1. *Nucleoproteins*.—Proteins derived mainly from the nucleus. Compounds of a simple protein with nucleic acid. Nucleohistone and nucleins. A large number of other nucleoproteins have been isolated from various organs. In many cases their neutral solutions are not coagulated by heat. All are precipitated by dilute acetic acid.

2. *Glucoproteins*.—Compounds of a simple protein with some form of carbohydrate. Mucins and mucoids. The mucins are insoluble in dilute acetic acid, and readily soluble in dilute alkalies. Their neutral solutions are not coagulated by heat. Some of them, *e.g.* ichthulin, contain phosphorus, and are therefore known as phosphoglucoproteins.

3. *Chromoproteins*.—The prosthetic group is a pigment. Hæmoglobin, hæmocyanin, phycoerythrin, and others.

III. Derivatives of Proteins, which still retain many of the characters of intact protein.

1. *Metaproteins*.—Acid and alkali metaproteins or albuminates.

¹ Gelatin is a derived "scleroprotein," being a product of the incomplete hydrolysis of collagen.

2. *Coagulated Proteins*.—Irreversible, insoluble products of proteins produced by the action of heat, prolonged action of alcohol or acetone, and by enzymes. Coagulated albumin, and globulin, fibrin, casein, and others.

3. *Proteoses*.—Products of the partial hydrolysis of proteins. Not coagulated by heat, precipitated by saturation of their solutions with ammonium sulphate.

4. *Peptones*.—Later products of the partial hydrolysis of proteins. Not coagulated by heat and not precipitated by saturating their solutions with ammonium sulphate. All give the biuret reaction, and the majority also give the xanthoproteic reaction.

5. *Polypeptides*.—These are compounds of amino acids of known structure. The majority are synthetic products.

The readily accessible proteins of egg white may be used as types of the proteins in general. Egg white is separated from the yolk, thoroughly whipped so as to break up the egg membranes, and filtered through calico, which has been previously freed from starch by thorough washing. The viscous fluid thus obtained is faintly yellow in colour, alkaline in reaction, and has a specific gravity of 1.045. It contains 10 to 13 per cent. of protein, the greater proportion of which consists of egg albumin.

A small quantity of the filtered egg white, on dilution with nineteen times its volume of water, yields a well-marked white precipitate—egg globulin—which readily dissolves on the addition of a few drops of a saturated solution of sodium chloride. If the greater part of the egg white be similarly diluted with water, and filtered from the precipitate of globulin, one obtains an approximately .5 per cent. solution of ovalbumin, together with other substances, to be studied later. This solution may be utilised for the following reactions:—

GENERAL PROTEIN REACTIONS

(WITH A .5 PER CENT OVALBUMIN SOLUTION).

The reactions for proteins may be divided into colour and precipitation reactions.

I. COLOUR REACTIONS.

These are dependent upon the presence of certain atomic groups in the protein molecule.

1. BIURET, or PIOTROWSKI'S REACTION.

Add to a small quantity of the aqueous solution of egg white about half its volume of strong caustic soda or potash solution, and one or two drops of very dilute cupric sulphate. A violet coloration results. Excess of copper sulphate renders the reaction indistinct, by lending to the solution a bluish tint which is not characteristic. On heating, the violet solution acquires a pink tint.

This reaction depends on the presence of at least two $\text{—CO—NH}_2\text{—}$ groups, either united directly with one another, or connected by a single C or N atom, or by one or more —CO.NH— groups.

2. MILLON'S REACTION.

To a small quantity of the egg albumin solution add an equal volume of Millon's reagent. A white precipitate forms, which on heating becomes red in colour and collects itself into coagula.

This reaction depends upon the presence of an aromatic radicle in the protein molecule. It is given by all substances which contain the group $\text{C}_6\text{H}_5\text{—}$, with at least one H replaced by OH, *e.g.* phenol, tyrosine, etc. Millon's reagent is a solution of mercury in nitric acid containing some nitrous acid.

3. XANTHOPROTEIC REACTION.

Add to a small quantity of egg albumin solution, nitric acid, until precipitation of the protein is complete. The white precipitate produced becomes yellow on heating, and gathers itself together into flocculent coagula. Cool the solution and add ammonia, caustic soda, or potash, in excess. The colour of the coagula changes to an orange. If the alkali be gradually added, a more or less distinct precipitate forms in the acid fluid—acid albumin—which dissolves in excess of the alkali, to form an orange-coloured

solution. This precipitate is more abundant, if excess of the acid has originally been added.

The reaction is due to the formation of nitro-substitution products from aromatic radicles in the protein molecule.

4. ADAMKIEWICZ'S REACTION.

Add large excess of glacial acetic acid to a small quantity of undiluted egg white. Heat the solution, cool, and let some concentrated sulphuric acid flow slowly down the side of the inclined test-tube. On standing, a reddish-violet colour appears at the line of junction between the two fluids.

This reaction is due to the presence of glyoxylic acid, present as an impurity in glacial acetic acid (Hopkins and Cole). The reaction probably indicates the presence of an indole or skatole radicle in the protein molecule.

5. HOPKINS' AND COLE'S MODIFICATION OF THE TEST.

To a small quantity of egg albumin solution add an equal volume of dilute glyoxylic acid (see Appendix), then at least one-third the total volume of concentrated sulphuric acid, and heat gradually. The fluid acquires a reddish-violet colour.¹

6. LIEBERMANN'S REACTION.

Add large excess of concentrated hydrochloric acid to a small quantity of undiluted egg white, and heat the solution to the boiling point. The solution acquires a violet colour. On standing, the tint changes to a brown, and a dark brown precipitate separates out.

This reaction is probably due to the presence of a carbohydrate and an aromatic radicle in the protein molecule. The furfural formed by the action of the acid on a carbohydrate radicle unites with an aromatic radicle to give the violet coloration. Skatole, however, when heated with concentrated hydrochloric acid, gives a similar reaction.

¹ A similar reaction is obtained when a dilute solution of formaldehyde (1 : 2500) is used in place of the glyoxylic acid solution, and the sulphuric acid added contains a trace of an oxidising agent such as ferric chloride or nitrous acid (ROSENHEIM).

II. PRECIPITATION REACTIONS.

1. HEAT COAGULATION.

Heat a small quantity of the aqueous egg albumin solution. A precipitate forms before the solution has reached the boiling point. On faint acidification with a drop or two of 1 per cent. acetic acid, the precipitate separates out more completely in the form of flocculi. Complete coagulation is insured if one-sixth its volume of concentrated sodium chloride solution be added to the egg albumin solution prior to heating. The coagulation of albumin by heat is an irreversible process, or, in other words, the precipitate of albumin will be found to have become permanently insoluble in water.

[Determination of the Temperature of Coagulation of Albumin.]

Place some 1 in 20 egg albumin solution in a test-tube, and render it very faintly acid with dilute acetic acid. Fit the test-tube with a cork having two holes, through one of which a thermometer passes. The egg albumin solution should completely cover the bulb of the thermometer. Fix the tube vertically by means of a clamp, and immerse it in water contained in a large beaker. Gradually heat the water in the beaker, stirring constantly, and note the temperature at which a cloud appears in the albumin solution.

2. PRECIPITATION BY CONCENTRATED MINERAL ACIDS.

E.g. nitric and hydrochloric acids. The precipitate produced dissolves only with difficulty in excess of the acids used.

Place some concentrated nitric acid in a test-tube. Then pour a little albumin solution slowly down the side of the inclined tube, so that it forms a layer above the acid. Note the opaque ring due to precipitated albumin at the plane of junction; then mix the fluids by shaking. On boiling the coagula which form, partially dissolve in excess of the acid.

3. PRECIPITATION BY NEUTRAL SALTS OF THE ALKALINE METALS AND OF MAGNESIUM.

For the following tests a solution of egg white should be used in which the globulin has been brought into solution by the addition of a few drops of concentrated sodium chloride solution. Since the quantity of egg globulin is small, blood serum diluted with four volumes of 0.9 per cent. sodium chloride may be used with advantage in the place of egg white for tests 3 (a) to (d).

(a) *To a small quantity of the solution gradually add finely-powdered magnesium sulphate, and continue adding it until some remains undissolved. The solution of the salt may be aided by shaking, and by heating the test-tube on the water bath to a temperature not exceeding 30°. After ten minutes, filter from the precipitate of globulin which forms, and heat part of the filtrate to the boiling point, then faintly acidify with dilute acetic acid. A coagulum of ovalbumin forms. To another portion of the filtrate add two drops of dilute hydrochloric acid in the cold; a precipitate of ovalbumin forms.*

This illustrates the influence of the reaction upon the precipitability of proteins.

(b) Saturation with sodium chloride also precipitates the globulin.

Saturate with NaCl, shake thoroughly and filter.

The albumin may be precipitated from the filtrate by the addition of a few drops of dilute hydrochloric acid, or in the form of a coagulum by heat.

Divide filtrate into two portions, heat one, and add a few drops of dilute acetic acid to the other.

(c) *To another portion of the solution add an equal volume of saturated ammonium sulphate solution. Shake. A precipitate of globulin appears. Filter, and saturate filtrate with finely powdered ammonium sulphate; a precipitate of ovalbumin forms.*

The precipitates of globulin or albumin, produced by saturation of their solutions with neutral salts, may be redissolved in dilute solutions of neutral salts or water respectively. They consist of unaltered proteins.

[(d) *Half saturate with anhydrous sodium sulphate at 30° (circa, 25 per cent.), egg globulin is precipitated. Completely saturate at 30°, ovalbumin is precipitated.*]

4. PRECIPITATION BY SALTS OF THE HEAVY METALS.

E.g. mercuric chloride, copper sulphate, or basic lead acetate.

N.B.—In solutions containing chlorides, phosphates, or sulphates, the last salt yields precipitates of lead chloride, phosphate, or sulphate.

(a) *To a small quantity of aqueous egg albumin solution add two or three drops of a solution of mercuric chloride. A heavy white precipitate—mercuric albuminate—forms, which readily dissolves on the addition of a small quantity of a saturated solution of sodium chloride. The mercuric albuminate may be reprecipitated from its solution in sodium chloride by the addition of a few drops of dilute hydrochloric acid.*

(b) *To a small quantity of aqueous ovalbumin solution add, drop by drop, dilute cupric sulphate solution as long as a bluish-white precipitate continues to form (ca., two to three drops). The precipitate dissolves in excess of caustic alkali to form a violet solution (Piotrowski, or biuret test).*

5. PRECIPITATION BY ALKALOIDAL REAGENTS IN ACID SOLUTION.

(a) *Hydroferrocyanic acid.*

Add to a small portion of aqueous egg albumin solution acetic acid up to approximately 2 per cent., and then, drop by drop, potassium ferrocyanide solution (1 in 20), shak-

ing after each addition, so long as the precipitate continues to increase in bulk. A voluminous precipitate forms.

The presence of neutral salt renders precipitation less complete.

(b) *Picric acid.*

Add to a portion of the aqueous solution a few drops of picric acid = precipitate.

(c) *Tannic acid.*

A precipitate forms on the addition of a few drops of tannic acid solution to the solution.

(d) *Trichloroacetic acid.*

Add to a small quantity of the solution a few drops of a 2 to 4 per cent. solution of trichloroacetic acid; a precipitate forms.

(e) *Potassio-mercuric iodide (Brücke's reagent).*

Acidify a small portion of the solution with dilute hydrochloric acid, and add a few drops of Brücke's reagent; a distinct precipitate forms.

[(f) *Metaphosphoric acid.*]

A precipitate also forms on addition of a few drops of a freshly prepared aqueous solution of metaphosphoric acid to the solution.

6. ALCOHOL.

To a small quantity of egg albumin solution add methylated spirit; a precipitate of albumin forms.

The precipitate, which is at first capable of re-solution in water, is rapidly converted into an insoluble modification or coagulum.

[7. ETHER.]

Slightly acidify with dilute sulphuric acid a small quantity of the aqueous egg albumin solution, then add half its volume of ether, and shake gently; a flocculent precipitate of egg albumin forms.

[PREPARATION OF CRYSTALS OF OVALBUMIN
FROM EGG WHITE.¹]

HOPKINS' METHOD.

To undiluted fresh egg white an equal volume of a saturated solution of ammonium sulphate is gradually added, the mixture being thoroughly whipped after each addition. After standing for about twelve hours in a stoppered vessel, the solution is filtered from the precipitate of egg globulin. The filtrate is alkaline in action, and has an ammoniacal odour. 10 per cent. acetic acid, either dissolved in water, or, better, in half-saturated solution of ammonium sulphate, is gradually added to the filtrate from a burette, the mixture being shaken after the addition of each drop. The addition of dilute acetic acid is continued until a very faint opalescence forms. The vessel is then stoppered, and allowed to stand for twelve to twenty-four hours. A copious precipitate will then have formed, which on microscopic examination will be found to consist of acicular crystals, free from amorphous material.²

Other dilute acids, e.g. $\frac{N}{5}$ sulphuric acid, may be used instead of acetic. If $\frac{N}{5}$ sulphuric acid be used, the acid solution should be made up with half-saturated ammonium sulphate solution instead of water.

[DEMONSTRATION OF THE COLLOID NATURE
OF ALBUMIN.]

Place a solution of egg albumin in 5 per cent. sodium chloride, either in a sausage tube dialyser, suspended in a tall cylinder containing distilled water, or in a ring dialyser. After twenty-four hours, test a portion of the dialysate for chlorides, by the addition of nitric acid and silver nitrate,

¹ These crystals may be demonstrated to the class.

² Serum albumin crystals may readily be obtained from the blood serum or oxalate plasma of horse's blood by this method.

and for proteins by any of the colour reactions. In the former case the result is positive, in the latter negative.

THE ALBUMINS (I.) AND GLOBULINS (II.) OF BLOOD SERUM.

Blood serum is a clear, or faintly opalescent, alkaline, slightly viscid fluid, having a more or less distinct yellow tint. It is frequently of a slight red colour, owing to the presence of dissolved oxyhæmoglobin.

(a) *To 20 c.c. water, acidified with three drops of 1 per cent. acetic acid, 1 or 2 c.c. of blood serum are added. On standing, a finely flocculent precipitate of serum globulin separates out from the solution.*

(b) *Prepare a saturated aqueous solution of magnesium sulphate. To this add a few drops of blood serum, a precipitate of serum globulin separates out.*

(c) *To a portion of blood serum add an equal volume of a saturated solution of ammonium sulphate. Filter off the precipitate of serum globulin which separates out. Heat a small portion of the filtrate; a coagulum of serum albumin forms.*

(d) *Saturate the remainder of the filtrate with finely powdered ammonium sulphate; a precipitate (e) of serum albumin forms. Filter off this precipitate. The filtrate, being protein free, will be found to give no xanthoproteic reaction.*

(e) *The precipitate of serum albumin dissolves readily in water. The solution thus obtained may be tested in the same way as that of egg albumin. The following are the chief differences which will be noted:—*

EGG ALBUMIN.

1. Precipitated by ether.
2. Readily precipitated by concentrated hydrochloric acid; only soluble with difficulty in excess.
3. Rapidly and easily precipitated by alcohol; precipitate rapidly becomes insoluble in water.

SERUM ALBUMIN.

1. Not precipitated by ether.
2. Precipitate which forms with conc. HCl dissolves readily in excess of the acid.
3. More slowly precipitated by alcohol; the precipitate, if quickly filtered off, is soluble in water.

Carry out these three tests with egg albumin and serum albumin solutions.

From the tests which have been carried out, tabulate the distinctive characters of albumins (I.) and globulins (II.) before going on to the tests for bodies derived from these (acid and alkali albumins, albumose, peptone).

ACTION OF ACIDS (III.) AND ALKALIES (IV.) ON EGG ALBUMIN.

III. ACTION OF ACIDS: ACID ALBUMINS.

(a) *To a small quantity of undiluted egg white add about one-third its volume of glacial acetic acid. Mix thoroughly. On standing, a jelly consisting of acid albumin forms.*

(b) *To half a test-tubeful of 1 in 10 aqueous solution of egg white add five to ten drops of a saturated solution of oxalic acid.¹ Keep the solution at a temperature of about 50° for a few minutes. Then gradually heat the solution to the boiling point. No coagulum results.*

(c) *Add to a portion of the cooled solution a few drops of litmus, and then, drop by drop, dilute caustic soda (circa, .2 per cent.); a precipitate of acid albumin forms, while the solution is still faintly acid.*

(d) *Saturate another portion of the acid albumin solution with sodium chloride or magnesium sulphate; a precipitate of acid albumin forms.*

IV. ACTION OF ALKALIES, ALKALI ALBUMINS.

(a) *To a small quantity of undiluted egg white add one-third its volume of a concentrated solution of caustic soda. Mix thoroughly; a jelly of alkali albumin (Lieberkühn's jelly) forms.*

¹ Oxalic acid is better than the dilute mineral acids usually employed for the formation of acid albuminates.

(b) Heat another portion of egg white with twice its volume of 10 per cent. caustic soda. Ammonia is given off. It may be detected by its odour, by the alkaline reaction with moistened red litmus paper, by the formation of ammonium chloride with the fumes from the bottle of concentrated hydrochloric acid, or by the blackening of a piece of filter paper moistened with mercurous nitrate solution. The quantity of ammonia given off is small, and therefore somewhat difficult to detect.

(c) Divide the alkaline solution into two portions. To one of these add a solution of lead hydrate in caustic soda = brownish-black precipitate of lead sulphide. To the other, add a dilute solution of sodium nitroprusside = a reddish-violet coloration. "Loosely combined sulphur."

(d) To half a test-tubeful of 1 in 10 aqueous solution of egg white add three or four drops of 10 per cent. caustic soda, and digest on the water bath at 40° to 50° for ten minutes. Then heat the solution gradually to the boiling point = no coagulation.

(e) Divide the cooled solution into two parts. To one of these add a few drops of litmus solution, and then, drop by drop, dilute acetic acid, until a precipitate forms. Excess of the acid redissolves the precipitate.

(f) Add dilute acetic acid to another portion of the alkali albumin solution, until it is nearly neutralised; then saturate with sodium chloride = a precipitate.

PROTEOSES (V.) AND PEPTONES (VI.).

These products of the digestion of proteins will be studied more fully in connection with gastric and pancreatic digestion. For the following reactions, a 5 per cent. solution (or weaker) of "Witte's peptone," in 5 per cent. sodium chloride solution, may be employed.

V. PROTEOSES.¹

(a) *Boil some proteose solution = no precipitate.*

(b) *To a small portion of the solution add, drop by drop, concentrated nitric acid, as long as the precipitate continues to increase. Then heat the solution to the boiling point. The precipitate dissolves, to form a yellow solution. On cooling under the water tap, the precipitate reappears.*

N.B.—Care must be taken not to employ excess of the acid.

(c) *Biuret reaction = pink, not violet coloration.*

(d) *Saturate part of the solution with sodium chloride = pp. of primary proteoses. Filter.*

(e) *Acidify filtrate with acetic acid = pp. of part of the deuterio-proteose (secondary proteose).*

VI. PEPTONES (WITH ALBUMOSES).

(a) *Render slightly acid with dilute acetic acid, and saturate the solution with ammonium sulphate = a precipitate of albumoses.*

(b) *Filter. The filtrate will give a more or less distinct yellow colour with nitric acid, but no precipitate. On rendering alkaline, the tint deepens to an orange.*

The biuret reaction in the filtrate can only be shown when a large excess of KOH or NaOH is added.²

The quantity of true peptone is usually very small.

The proteins which have been examined are the so-called simple native proteins and bodies of protein nature derived from them.

There are others which have another radicle, *e.g.* a nuclein, or hæmatin (as in hæmoglobin), or a carbohydrate radicle (as

¹ The term proteoses is given to this class of substances. The proteoses are then divided into sub-classes having names derived from those of the proteins from which they take their origin, *e.g.* albumoses, globuloses, fibrinoses, gelatoses, etc.

² The ammonia set free dissolves the cupric hydrate, forming a dark blue solution, which interferes with the biuret reaction. The presence of free caustic soda or potash is essential for the latter.

in mucin), united to the simple protein. These will be studied later in connection with the tissues in which they occur.

There is a group of proteins, containing members which are difficult to classify otherwise, namely, the albuminoids. As examples gelatin and keratin may be taken.

ALBUMINOIDS OR SCLEROPROTEINS.

I. GELATIN.

Commercial gelatin always contains traces of other proteins, from which it may be partially freed by washing with cold water. After washing with cold water, in which the gelatin swells up, but does not dissolve, the gelatin is well pressed.

A small quantity of gelatin, on heating with water, dissolves. On cooling, the solution, if sufficiently concentrated, gelatinises. If the solution be boiled for some time, it loses the power of forming a jelly on cooling.

A dilute solution will serve for the following reactions:—

I. COLOUR REACTIONS.

(a) PIOTROWSKI'S REACTION (see p. 51), bluish-violet colour.

Do this test.

(b) XANTHOPROTEIC REACTION.

To a small quantity of the gelatin solution, add three or four drops of concentrated nitric acid. No precipitate forms. On heating, the solution acquires a very faint yellow tint. Cool the solution and render alkaline with ammonia = slight deepening of the yellow tint.¹

(c) MILLON'S REACTION.

To a small portion of the gelatin solution, add Millon's reagent = white precipitate. On boiling, the precipitate, as a rule, becomes faintly red.

¹ This reaction is much less marked in the case of gelatin than with other protein substances.

The reddening is possibly due to traces of other proteins, present as an impurity in the gelatin.¹

II. PRECIPITATION REACTIONS.

(a) *Saturate portions of the solution with magnesium sulphate and with ammonium sulphate. Precipitates form in both cases.*

(b) TANNIC ACID.

With tannic acid a copious precipitate forms.

(c) *Mercuric chloride does not precipitate gelatin from a dilute neutral solution, but in the presence of dilute hydrochloric acid a copious precipitate forms.*

(d) *Lead acetate does not precipitate gelatin.*

(e) *Acetic acid and potassium ferrocyanide also do not produce a precipitate, or only a very slight one.*

(f) *To a small quantity of the solution of gelatin add bromine water; a copious precipitate forms. The precipitate being soluble both in excess of bromine water and of gelatin is frequently difficult to obtain.*

II. KERATIN.

The albuminoid or scleroprotein keratin is distinguished by its high resisting power to the action of chemical agents.

For the following tests horn shavings may be employed:—

1. *Note its insolubility in hot and cold water and in dilute acids. By prolonged boiling with acids it is hydrolysed (Part II.).*

2. *Heat a small quantity with 10 per cent. caustic soda. It only undergoes very partial solution. Add a drop of lead acetate. The horn shaving blackens, the solution becomes of a brownish-black colour, and, on standing, a precipitate of lead sulphide separates out.*

¹ Although tyrosine cannot be obtained from pure gelatin, it is probable that even the purest specimens give a slight Millon's reaction.

Keratin is characterised by the large proportion of both loosely and firmly combined sulphur which it contains.

3. *Suspend a small portion in water, add a few drops of HNO_3 , and heat. The shaving acquires a yellow colour, which darkens to an orange on rendering the cool fluid alkaline by the addition of ammonia.*

4. *Keratin also gives Millon's reaction when heated with excess of the reagent. This colour reaction is frequently not quite typical in tint.*

Appendix to Chapter on Proteins

I. Removal of Crystalloids from Blood Serum by Dialysis.

1. Preparation of Collodion Tubes or Sacs.

Dissolve 8 to 10 grms. of celloidin or pyroxylin in 100 c.c. of a mixture of equal volumes of absolute alcohol and anhydrous ether. The process of solution may be facilitated by shaking. When the pyroxylin has completely dissolved, the solution is allowed to stand until air bubbles have risen to the surface. A wide glass centrifuge tube of about 50 c.c. capacity is filled with the collodion solution, from which any air bubbles may be removed by centrifugation. The tube is then inverted over a wide-mouthed bottle and allowed to drain. When drainage is complete the collodion film is allowed to dry until it no longer adheres to the finger. If desired, the drying may be hastened by aspirating a current of air through the tube. The tube is then filled with water, left immersed in water for an hour, and finally washed thoroughly with several changes of water. The upper margin of the collodion membrane is then separated by cutting round the rim of the tube with a sharp knife, and the membrane, while immersed in water, is gently separated from the walls of the tube by allowing water to pass between it and the glass. The collodion sac is filled with water, and while immersed in a vessel of water is slipped over the narrow end of a rubber stopper previously fitted with a short piece of wide glass tube passed through an opening in the stopper. The membrane is then tied in position with a piece of linen tape. The water is then drained out, and the collodion sac, after being charged with blood serum to which a little toluene is added, is immersed in a small beaker of distilled water, the glass tube from which it is suspended being securely clamped.

2. Separation of Serum Globulin and Serum Albumin.

After dialysis has continued for one or two hours, the fluid in the outer vessel is tested for chlorides with silver nitrate. A small portion should also be tested for protein by means of the biuret reaction. If the latter reaction be negative, no leakage is occurring, and the dialysis is continued, the distilled water in the outer vessel being frequently renewed. The dialysis is continued until the dialysate is free from chlorides. The contents of the collodion sac are

then transferred to a centrifuge tube and the precipitate of globulin separated by centrifugation. The clear, supernatant fluid containing serum albumin and pseudo-globulin is then poured off. Add an equal volume of saturated solution of ammonium sulphate to a portion of the clear fluid, a protein known as pseudo-globulin is precipitated. The filtrate from this precipitate contains serum albumin which may be identified by the reactions given elsewhere. The globulin, which is precipitated by dialysis, and is therefore insoluble in water, is known as euglobulin. It may be purified by dissolving it in a 2 per cent. solution of sodium chloride filtering, and again precipitating the euglobulin from the filtrate by half-saturation with ammonium sulphate. The protein known as serum globulin, therefore, consists of two proteins—euglobulin and pseudo-globulin.

II. Adsorption of Protein by fine Suspended Particles. Kaolin Method of freeing a Solution from Protein.

10 c.c. or grms. of blood serum, or oxalate plasma, are placed in a flask of about 250 c.c. capacity, diluted with about 130 c.c. of water (accurately measured), and faintly acidified with dilute acetic acid (about 10 per cent.), accurately measured from a burette until the turbidity, which first appears, just commences to clear. 30 grms. of kaolin are then gradually added, the solution being vigorously shaken after each addition. When all the kaolin has been added, the mixture is shaken occasionally and allowed to stand for about 15 minutes. It is then filtered through close filter-paper. The filtrate should be clear, perfectly colourless, and free from protein. If the slightest trace of pigment be present, or if an aliquot portion of the filtrate after being rendered faintly alkaline and concentrated on the water bath give the biuret reaction, the adsorption has been incomplete. The filtrate is frequently turbid from suspended particles of kaolin. If this occur, it should be returned to the filter-paper until a clear filtrate is secured.

When this method is employed for freeing solutions from proteins preparatory to the estimation of sugars, an accurately measured aliquot portion of the filtrate may be concentrated on the water bath to the volume required.

III. Preparation and Properties of some Conjugated Proteins.

1. Ovomuroid.

Dilute the whites of 2 or 3 eggs with 4 volumes of water, and add this solution to 6 volumes of boiling water faintly acidified with acetic acid. Filter off the coagulum of ovalbumin and ovoglobulin, concentrate the filtrate to about one-fifth of its original

volume, and precipitate the ovomucoid with about 5 volumes of alcohol. The ovomucoid is filtered off, washed with alcohol and ether and dried in vacuo. It may be purified by dissolving it anew in boiling water, filtering, and again precipitating with alcohol. A little more than 1 gm. is obtained from 3 eggs.

- (1) Dissolve a small quantity in boiling water.
- (2) Add 2 or 3 drops of nitric acid to part of the cooled solution. A yellow colour deepened to orange by ammonia is obtained, but no precipitate.
- (3) The biuret reaction gives a positive result.
- (4) On heating with Millon's reagent the solution becomes red, but yields no immediate precipitate.
- (5) Liebermann's test yields a positive result.
- (6) On applying the glyoxylic acid test no distinctly positive reaction occurs.
- (7) The solution gives a positive result with Molisch's test.

HYDROLYSIS OF THE OVOMUCOID.

The remainder of the ovomucoid is gently boiled for 3 to 4 hours with 20 c.c. of N/1 hydrochloric acid in a flask provided with a reflux condenser. The fluid is then neutralised, filtered, and tested with Fehling's solution. Reduction is obtained. The reducing substance appears to be glucosamine.

2. Preparation and Properties of the β -Nucleoprotein of the Pancreas (Hammarsten).

250 grms. of finely minced pancreas are added to a litre of boiling water. The mixture is then heated on the water bath for about half an hour, and filtered while hot. The nucleoprotein is then precipitated from the filtrate by the gradual addition of 30 per cent. acetic acid (10 to 15 c.c.). The precipitate is allowed to settle or is separated by the centrifuge, washed repeatedly with water followed by alcohol and ether, and dried in vacuo. The nucleoprotein may be purified by dissolving it in dilute ammonia, filtering, and precipitating the protein from the filtrate by acetic acid.

1. The nucleoprotein contains 4.48 per cent. of phosphorus, which may be estimated by Neumann's method.

2. Other portions of the nucleoprotein will be found to give well-marked positive pentose reactions with phloroglucinol and orcinol. When heated with acid it yields furfural. The percentage of pentose present in the pancreas is much greater than that found in any other organ or tissue. The pentose has been identified as d-ribose (Levene and Jacobs).

If sufficient nucleoprotein be available, the purine base—guanine—which is another product of the decomposition of the nucleoprotein may be isolated. One part of nucleoprotein is heated for some hours on the water bath with 30 parts of 3 per cent. sulphuric acid. The solution is then nearly neutralised with barium hydrate and filtered while hot. On cooling, some guanine sulphate separates out. More guanine sulphate may be obtained by washing out the barium sulphate

precipitate with hot dilute sulphuric acid. The guanine sulphate is filtered off, the filtrate is neutralised with barium carbonate, the solution again filtered and concentrated on the water bath. This solution will be found to contain pentoses, which may be identified by the colour reactions and the ordinary reduction tests. Guanine is the only purine base, which has been isolated from the decomposition products of the nucleoprotein of the pancreas.

3. Preparation of Sodium Nucleate from the Thymus (Neumann's method).

Sodium nucleate is a product of the decomposition of a nucleoprotein of the thymus, known as nucleohistone. The latter is split up by the action of hot dilute caustic alkali into sodium nucleate and alkali albuminates. The alkali albuminates, being salts of a much weaker acid than nucleic, are decomposed by acetic acid, and the greater part of the protein precipitated, while the sodium nucleate is not decomposed by acetic acid, and therefore remains in aqueous solution. The latter is then precipitated from the concentrated filtrate by alcohol, any acid metaprotein being also precipitated. The sodium nucleate precipitate retains its solubility in water, while any acid metaprotein which may be present is coagulated by the alcohol and thus becomes insoluble in water.

500 grms. of the thymus (calf) are placed in boiling water rendered slightly acid with acetic acid. The hardened gland is then withdrawn, finely minced, and gradually added to a boiling solution of 100 grms. of sodium acetate and 17 grms. of caustic soda in rather more than 1 litre of water (about 1100 c.c.). The flask containing the mixture is then immersed in a boiling water bath, shaken occasionally, and heated for fully 2 hours. The solution is rendered slightly acid to litmus paper by the addition of 50 per cent. acetic acid (about 50 c.c.), heated anew to the boiling point, and filtered while hot, preferably with a hot water funnel. The filtrate is concentrated to about 500 c.c. on the water bath, and the concentrated solution while still warm (40° to 50°) is poured into about 750 c.c. of alcohol. The sodium nucleate precipitate is allowed to settle overnight, and is then washed by decantation with 80 and 95 per cent. alcohol. The coherent mass of sodium nucleate after the alcohol has been expressed, is dissolved by heating on the water bath with about 300 c.c. of water. The resultant solution is filtered off from a deposit, which separates out, and the sodium nucleate again precipitated with alcohol, a little sodium acetate being added to facilitate the precipitation. The nucleate may be purified by dissolving again in water and repeating the precipitation with alcohol, some sodium acetate being also added. The final precipitate is washed by decantation with increasing strengths of alcohol, dehydrated by grinding it up with absolute alcohol in a mortar, and dried in vacuo over calcium chloride. The yield is about 16 grms.

Carry out the following exercises with an aqueous solution (1 per cent.) of sodium nucleate:—

(1) The cold solution is acid to phenolphthalein, and practically neutral to litmus. Immerse a piece of red litmus paper in a small

portion of the solution, and heat. The paper gradually becomes blue owing to hydrolysis.

(2) Acidify another portion of the solution with acetic acid. The solution remains clear. Acidify other portions of the solution with dilute sulphuric, hydrochloric, or nitric acids, a white precipitate of nucleic acid separates out. On heating the suspension of nucleic acid in dilute sulphuric or hydrochloric acids, it dissolves to form a clear solution.

(3) Add a few drops of dilute copper sulphate to another portion of the sodium nucleate solution, a precipitate of cupric nucleate separates out. On rendering this solution alkaline with caustic soda no biuret reaction is obtained. Millon's reagent gives a white precipitate of mercuric and mercurous nucleates, which does not change colour on heating.

(4) Add a small quantity of serum albumin to another portion of the nucleate solution. Then acidify by the gradual addition of acetic acid; a compound of albumin and nucleic acid, which separates out, dissolves readily in excess of acetic acid. This compound may be regarded as a synthetic nucleoprotein.

(5) If a portion of the sodium nucleate solution (preferably about 2-3 per cent.) be examined with the polarimeter, it will be found to be dextrorotatory. Calculate the specific rotatory power of sodium nucleate from the observed rotation. The specific rotation falls on dilution with water and more rapidly on dilution with alkali.

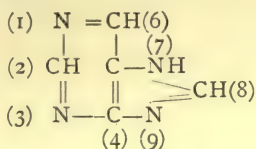
(6) Add about 5 volumes of alcohol to 1 volume of the sodium nucleate solution. If the sodium nucleate be pure, no precipitate is thrown down. On adding a few crystals of sodium acetate to this solution a white flocculent precipitate of sodium nucleate separates out.

(7) Place 20 c.c. of 2 N. sulphuric acid in a small flask, heat nearly to the boiling point, and gradually add 2.5 grms. of finely powdered sodium nucleate. Attach a glass tube as reflux condenser, and gently boil the solution for 4 hours. The solution becomes dark brown owing to the formation of humin substances, and contains lævulinic and formic acids, which may be separated from it by extraction with ether, and then purified by distillation. These are derived from a hexose precursor.

(8) Dissolve 5 grms. of barium hydrate in 50 c.c. of warm water and add this solution to the products of hydrolysis of sodium nucleate. Heat the mixture to the boiling point, filter off the barium sulphate, neutralise with ammonia the filtrate which still contains free sulphuric acid, and add excess of ammonia until the fluid contains about 2 per cent. Filter off the precipitate of impure guanine, which separates out, wash the precipitate with dilute ammonia (about 1 per cent.), and add the washings to the rest of the filtrate. Add to one small portion of the filtrate a small quantity of magnesia mixture. A precipitate of ammonium magnesium phosphate separates out. Acidify another small portion with acetic acid, add uranium acetate, and heat. A precipitate of uranyl-phosphate forms. On adding ammoniacal silver nitrate to the remainder of the filtrate, a flocculent precipitate, insol-

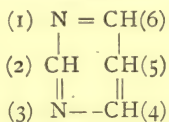
uble in excess of ammonia, consisting mainly of the silver compound of adenine with a smaller quantity of that of guanine separates out. The chlorides of these bases may be prepared from the silver salts by decomposing the latter with dilute hydrochloric acid, filtering off the precipitate of silver chloride, and concentrating the filtrate to dryness, preferably by distilling off the excess of water and acid in vacuo.

The following are the hydrolytic products of thymus nucleic acid :— phosphoric acid, guanine, adenine (both purine bases), thymine and cytosine (both pyrimidine bases), and lævulinic and formic acids, both derived from a hexose. The mother substance of the purine bases has the following structure :—



Guanine is 2-amino-6-oxypurine, xanthine, derived from guanine by replacing NH_2 by OH , is 2-6-dioxypurine, adenine is 6-aminopurine, hypoxanthine 6-oxypurine, and uric acid is 2-6-8 trioxypurine.

The mother substance of the pyrimidine bases has the structure :—



Cytosine is 2-oxy-6-aminopyrimidine, and thymine is 5-methyl-2-6-dioxypyrimidine. The silver compounds of these bases are insoluble in water ; but soluble in excess of ammonia.¹

¹ The reader is referred to "Nucleic Acids," by W. Jones. (Longmans, Green & Co., London.)

CHAPTER V

ANIMAL AND VEGETABLE FOOD STUFFS

ANIMAL FOOD STUFFS

AMONG these, the most important are MEAT, MILK, and EGGS. The detection of the more important constituents of meat is given under "Muscle" on pp. 193 to 196, while milk is described on pp. 188 to 192.

Eggs.

In order to separate the white from the yolk, break the shell carefully, tear through the chalazae, and allow the mass of the white to flow away into a vessel. The white still adhering to the yolk may be removed (as in quantitative work) by rolling the latter carefully in a glass beaker which has been slightly moistened by the breath.

The main constituents of egg white are the proteins, which have been already described (p. 69). The traces of reducing carbohydrates and fat are only of slight importance, and methods for their detection will not be described.

YOLK OF EGG.

Take the yolks of about ten eggs in a large, thick glass beaker, whip up thoroughly, and pour into wide-necked stoppered bottle; add about 200 c.c. of ether, and place in bottle-shaker for about half an hour. Pour off ethereal extract, add fresh ether, and repeat the shaking. Repeat the process until the ethereal extracts are almost free from colour. Now drain off excess of ether from residue by placing latter on stretched muslin, squeezing out as much ether as possible. Keep the collected ethereal extracts, then expose residue in thin layer to atmosphere, and blow current of air across it until ether has evaporated. Place residue in similar

wide-necked bottle, add 500 c.c. of 10 per cent. NaCl, and extract in bottle-shaker for three to four hours, or longer. Leave mixture standing overnight. Extract for short time again in morning, and then filter.

Pour the filtrate in portions into tall cylinders containing slightly acidified (acetic acid) distilled water. Shake thoroughly.

If the precipitate of VITELLIN does not separate out properly, pass through CO_2 . The precipitate separates out in fine flocculi. When it has settled, siphon off the supernatant fluid, and collect the precipitates in the various cylinders. Add about 500 c.c. of water; shake; then add, drop by drop, dilute NaOH until the precipitate dissolves.

[VITELLIN.¹]

This alkaline solution of vitellin may be given out to the class.

TESTS.

(a) *Do some of the colour reactions for proteins, especially biuret and Millon.*

(b) *Add, drop by drop, very dilute acetic acid until precipitate appears.*

(c) *Incinerate this precipitate with Na_2CO_3 and KNO_3 . Extract ash with dilute HNO_3 , add excess of ammonium molybdate, and warm. A yellow precipitate of phosphomolybdate of ammonium forms.*

This shows that this protein (which has been freed from lecithin? and phosphates) contains phosphorus.

(d) *Digest some of the precipitate with pepsin HCl; an indigestible residue, containing a large percentage of phosphorus, is left.*

To this the name of para- or pseudo-nuclein has been given.² The fluid of course gives the reactions for albumoses.

¹ The more characteristic tests of vitellin and lecithin may be shown to the class.

² It is better to use the term phospho-proteins for such bodies as vitellin and caseinogen (including casein).

[LECITHIN, CHOLESTEROL, FATS, and LUTEIN.]

These are present in the collected ethereal extracts. Distil off the ether until a solution of deep yellow tint is left. Keep a small quantity of this for spectroscopic examination.

Note the position of the bands (LUTEIN).

Distil off the remainder of the ether from the rest of the extract.

The residue contains lecithin, fat, and cholesterol, but no phosphates.

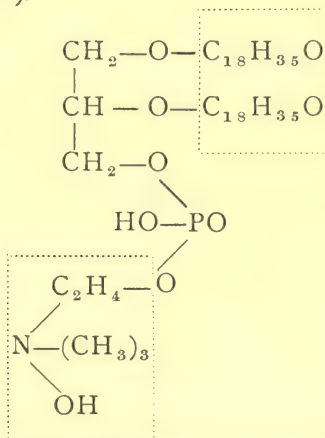
Add some saturated baryta water to the residue, shake, boil on oil or sand bath for one hour, with upright condenser; saturate with CO_2 , and filter while hot.

The filtrate contains glycerophosphate of barium and choline; the residue (C), barium soaps and cholesterol.

Evaporate down filtrate to dryness on water bath. Extract residue with absolute alcohol.

The alcoholic solution (A), and the residue (B), after extraction with alcohol, may be given out to each student to test for the decomposition products of lecithin.

DECOMPOSITION PRODUCTS OF LECITHIN (DISTEARYL-LECITHIN).



The CHOLINE is dissolved by the alcohol.

The alcoholic solution (A).

Precipitate by adding an alcoholic solution of platinic chloride (10%). Filter, dissolve precipitate in water, and allow crystals of cholin platinic chloride to separate out. Examine microscopically, and sketch the large orange-red crystals.

The residue (B).

Incinerate the residue, which contains glycerophosphate of barium, with Na_2CO_3 and KNO_3 , and test for phosphate in the usual way.

The residue (C).

This residue, which contains *cholesterol* and the barium salts of the fatty acids, may be examined in the following way :—

Add water, shake, acidify with HCl , and pour into separating funnel. Then add about an equal quantity of ether, and shake. Run off the aqueous solution, and to the ethereal one add sufficient NaOH to give a distinct alkaline reaction; then run off the alkaline watery solution, which may be tested for soaps. Distil off the excess of ether from the ethereal extract, and test for cholesterol (see p. 149).

VEGETABLE FOOD STUFFS.

The most important vegetable food stuff with which we have to deal is flour, especially in the form of bread preparations. It is therefore of importance to carry out some of the more distinctive tests suitable for its detection.

Flour.

Flour is ground wheat freed more or less completely from bran. It contains about 13 per cent. of water, 60 to 70 per cent. of starch, 9 to 10 per cent. of protein, about 1 to 2 per cent. of fat, and yields about 1.5 per cent. of ash. Five proteins have been isolated from flour (T. B. Osborne). The gluten, which constitutes about 8 per cent. of the flour, consists of two proteins—gliadin (about 4 per cent.) and glutenin

(about 4 per cent.) Gliadin is insoluble in water, but readily soluble in dilute alcohol (about 70 per cent.). Glutenin is insoluble in water, dilute neutral salt solutions, and in dilute alcohol, but soluble in dilute alkalies.

1. *Add a small quantity of water to some flour in a porcelain basin, and mix thoroughly so as to form a dough. Then place in a piece of muslin, and knead the mass in a basin containing water. The starch grains pass through the muslin, while an insoluble sticky mass, gluten, is retained.*

2. *Heat the aqueous extract to the boiling point, cool, and test a portion with iodine.*

A blue colour results, owing to the formation of iodide of starch.

3. *Test another portion for glucose (Trommer or Fehling). No reduction.*

4. Tests with gluten—

(a) *Suspend a little of the insoluble gluten in water, add a few drops of HNO_3 , and heat. The insoluble mass becomes yellow. Cool, and render the fluid alkaline with ammonia. The colour deepens to an orange.*

(b) *Suspend a small quantity in Millon's reagent, and heat.*

The mass acquires a reddish tint.

[5. *Place a small quantity of flour or gluten in a test-tube, add about 5 times as much 70 per cent. alcohol, mix thoroughly, and heat on the water bath to about 60° for half an hour, hastening the extraction by shaking the mixture frequently. Allow the undissolved material, which consists mainly of starch and glutenin (with some undissolved gliadin if flour has been used, or mainly of glutenin, and some gliadin if gluten alone has been extracted), to settle, and filter off the supernatant alcoholic solution through a filter paper moistened with 70 per cent. alcohol. Pour a little of the alcoholic filtrate into excess of distilled water, a precipitate of gliadin separates out.*

Apply the protein colour reactions to this suspension and note the results.]

[6. *Flour also contains a small quantity (0.3 per cent.) of an albumin known as leucosin, which may be extracted with cold water, and a globulin (0.6 per cent.), which may be obtained by extracting the flour with dilute neutral salt solution (10 per cent. sodium chloride).]*

A number of other cereals are important food stuffs.

Ground Oats or Oatmeal contains over 50 per cent. of starch, more protein (about 12 per cent.), and fat (about 6 per cent.) than flour. The chief proteins are a gliadin, a glutenin, a crystalline globulin soluble in 10 per cent. sodium chloride known as avenin, and an albumin.

Barley contains about 70 per cent. of carbohydrate, 11 to 12 per cent. of protein, about 4 per cent. of which is soluble in dilute alcohol, 2 per cent. soluble in salt solution, 0.3 per cent. soluble in water, and about 4.5 per cent. soluble only in dilute alkalis, and about 1.7 per cent. of fat.

On account of the physical characters of their proteins, oatmeal and ground barley are not so well adapted for the making of bread as flour.

Rice contains about 78 per cent. of starch, much less protein (about 7 per cent.) than the other cereals, and only traces of fat.

Bread.

Heat some bread crumb with water, and filter.

1. *Test the filtrate for starch by the iodine reaction, and for glucose by Trommer or Fehling.*

Reactions are positive.

2. *Perform the xanthoproteic and Millon's reactions with the residue, in the same way as for gluten.*

Results are positive.

Potato.

The uncooked potato contains about 76 per cent. of water, 20 per cent. of starch, traces of protein, asparagine (about 0.3

per cent.), solanin (a toxic glucoside), and other nitrogenous substances, and about 1 per cent. of ash, fully half of which consists of potassium salts. About 56 per cent. of the total nitrogen is derived from amino-acids, chiefly the amide, asparagine, with smaller quantities of leucine and tyrosine. The potato also contains traces of oxalic, citric, and succinic acids.

1. *Scrape the cut surface of a potato as finely as possible with a knife, transfer the scrapings to a small beaker of water, stir well, and strain through fine muslin into another beaker. Allow the white deposit of starch to settle and pour off the supernatant fluid. The starch may be purified by repeating this process. Examine a small quantity of the sediment microscopically and note the colour change with iodine. Heat another portion of the sediment with water. The starch dissolves to form an opalescent solution, which may be examined as already described.*

2. *Enclose 4 or 5 potatoes in a piece of calico, previously well washed and dried, place in a filter press and express the juice. Filter this fluid, test the filtrate with litmus paper, then raise the fluid to the boiling point. Filter off the coagulum of protein, which separates out. Small portions of the coagulum may be removed from the filter paper with a spatula and identified as protein by the biuret, Millon's, and xanthoproteic reactions. The filtrate contains mainly asparagine with smaller quantities of leucine and tyrosine. The unheated juice contains oxidising enzymes.¹*

Peas and other Leguminous Seeds such as Lentils and Beans.—These are important food stuffs containing large quantities of starch, and a much higher percentage of protein than the cereals. Dried peas contain 20 to 25 per cent. of protein, about 55 per cent. of starch, about 2 per cent. of fats,

¹ For an account of these the reader is referred to "Practical Plant Biochemistry," by M. W. Onslow, pages 87-117.

and yield 2 to 3 per cent. of ash. The chief proteins are globulins, soluble in 10 per cent. sodium chloride. There are also smaller quantities of albumin and a proteose. As contrasted with cereals, the absence of prolamines or proteins soluble in alcohol is noteworthy.¹

¹ The reader is referred to *loc. cit.* in note 1, page 134, for the methods of extracting the proteins of the pea.

CHAPTER VI

THE DIGESTIVE SECRETIONS

GENERAL CHARACTERS OF DIGESTION

THE process of hydrolysis, as a result of which the organic food stuffs are split into readily diffusible substances, is known as digestion. This disintegration of the food stuffs into simpler units is due to the action of substances known as enzymes. The latter may be defined as catalysts produced by living organisms, which are specific in the sense that the action of each is confined to certain substances or groups of substances known as substrates.

The enzymes are colloids of a highly labile character, being readily rendered inactive by many physical and chemical agents. For its full activity each enzyme requires an optimal temperature and definite reaction or hydron concentration. They all are more or less rapidly destroyed when the temperature of their solutions is raised above the optimal point. All are destroyed by boiling their solutions, although, when in the dry state, many resist temperatures considerably over 100° . Their activity diminishes when the temperature falls below the optimal point, and is temporarily suspended at 0° .

In consequence of their unstable character, their isolation from solutions containing other colloids is a problem of great difficulty, which is still far from being completely solved. They are soluble in water, dilute neutral salt solutions, and in glycerol diluted with water. They are precipitated by saturation with ammonium sulphate, and are generally carried down as adsorption compounds with amorphous

precipitates of other colloids. They also tend to be adsorbed on the surface of particles in fine suspensions.

The enzymes are characterised essentially by the changes which they produce in definite substrates. The actions of many enzymes may be facilitated by certain specific substances known as co-enzymes, or hindered by certain products of living cells known as anti-enzymes. In order to distinguish the chemical actions due to enzymes from those due to living organisms, advantage is taken of the fact that living protoplasm is more readily destroyed than enzymes by dilute solutions of antiseptic substances. Such feeble antiseptics as toluene, xylene, chloroform, thymol, and salicylic acid prevent the growth of bacteria, and only slightly impair the action of enzymes.

The enzymes are stored as inactive precursors, zymogens, or pro-enzymes within the gland cells which secrete them. The zymogens are converted into enzymes either during, or more frequently after, secretion by means of certain activating agents known as kinases.

The chemical reactions due to enzymes are generally reversible, although the products of reversion are frequently different from the original substrates. Enzyme actions are consequently usually incomplete, a position of equilibrium being reached in which the opposite reactions of hydrolysis and synthesis are occurring at the same rate, the exact position of equilibrium varying for different enzymes, and according to experimental conditions. If the products of hydrolysis be removed from the solution, *e.g.* by dialysis, or their concentration be reduced by dilution, the position of equilibrium may be made to approximate more or less nearly to complete hydrolysis. Thus the conversion of urea into ammonium carbonate by the enzyme urease becomes practically complete, if the volatile products of the action be removed from the solution. The inhibitory effect of the products of enzyme action has also been proved to be a specific one in a number of cases. For details regarding the latter subject, as well as the whole question of the laws regulating the velocity of enzyme reactions, the reader is referred to "The Nature of Enzyme Action," by W. M. Bayliss.

Nomenclature of the enzymes.—Various suggestions have been made to facilitate the classification of the enzymes by the adoption of a consistent method of nomenclature. This has been partially secured by the addition of the termination-*ase* to the root of the term applied to the substrate. Thus, an enzyme which hydrolyses starch (*amylum*) is known as amylase. Other examples are the terms lactase, maltase, lipases, and many others. Many of the names originally given to well-known enzymes are still retained, since they have become familiar from usage. The action of an enzyme is usually indicated by the termination *-lytic*, or better, *-clastic* (H. E. Armstrong) added to the root of the term applied to the substrate. Thus enzymes acting on proteins are known as proteolytic or proteoclastic.

Saliva.

The mixed secretions of the human salivary and buccal glands known as saliva have a specific gravity of 1.001 to 1.003, and contain 0.36 to 0.45 per cent. of solids, rather more than half of which consist of inorganic salts. The freezing-point of saliva varies from -0.20 to -0.43 . The normal reaction is slightly alkaline to litmus but acid to phenolphthalein. The optimal reaction for the enzyme (ptyalin) of saliva is $P_H = 6.7$ to 6.8 . The reaction may occasionally be faintly acid to litmus owing mainly to the presence of lactic acid arising from decomposition of residual carbohydrates of the food. The total daily secretion is very variable (500 to 1500 c.c.). The saliva contains in suspension a variable number of formed constituents, such as desquamated epithelial cells, leucocytes, etc. It may be freed from the latter by filtration.

1. Test *reaction* with litmus paper—alkaline.

2. *To a small quantity in a test-tube add a few drops of acetic acid—a stringy precipitate of mucin separates out.* Mucin is a complex protein (glucoprotein) containing a carbohydrate radicle, and hence, on boiling with dilute mineral acid solution, a CuO reducing body (glucosamine) is formed. Unlike other compound proteins—nucleo-albumin—it is not easily dissolved by acetic acid.

[3. Boil filtrate after separation of mucin, and if saliva contains albumin, that substance will be coagulated. The quantity of coagulable protein is so small that its detection is difficult.]

4. Biuret, xanthoproteic, and Millon's reactions—positive = mucin or albumin or both. All the reactions are faint.

5. Saliva often contains alkaline sulphocyanates, which, on addition of ferric chloride, may be detected by the formation of a red coloured solution (ferric sulphocyanate).

TEST.

Add a drop of HCl to a small quantity of saliva, and then, drop by drop, dilute ferric chloride (shaking after each addition) until solution becomes red in colour. Compare tint with acid ferric chloride solution alone.

This reaction is given more distinctly by the saliva of smokers than by that of non-smokers.

6. The most important characteristic of saliva is the power which it possesses of transforming polysaccharides, such as starch or glycogen, into dextrin and maltose. The first product of the action is amidulin, or soluble starch; from this erythro-dextrin and maltose are formed; and from erythro-dextrin, achroö-dextrin and maltose. After prolonged action, about 80 per cent. is in the form of maltose or, what used to be regarded as an allied disaccharide, isomaltose. In order that the action may be a marked one, the reaction of the mixture should be neutral or slightly acid, and the temperature about 40° C. The action depends upon the presence of the enzyme, *ptyalin*, the amylase of saliva.

TESTS.

With a glass rod put a series of drops of iodine solution on a porcelain slab.

PREPARATION OF THE STARCH MUCILAGE.

Take about 5 grms. of finely powdered starch in a mortar, add a small quantity of water, sufficient to make a thick paste

on admixture. Then gradually add this paste to half a litre of boiling water, stirring constantly. The flask must be frequently shaken to prevent the starch settling at the bottom of the flask. Small quantities are to be given to each student.

Diluted saliva may be obtained by washing out the mouth thoroughly with a small quantity of lukewarm water. The contents of the mouth are then transferred to a beaker, filtered, and the filtrate is used for (b).

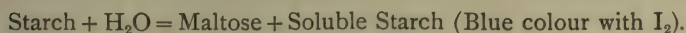
(a) In the first place, apply a drop of the starch mucilage to one of the iodine drops = blue iodide of starch.

(b) Take some starch mucilage in a test-tube and add about one-quarter the amount of diluted saliva to it. Put glass rod in test-tube. Place tube in water bath at 40° C. for a few seconds, then take out drop of mixture on glass rod, and apply it to one of the iodine drops. Probably reddish-violet colour of iodide of erythro-dextrin will appear, most likely along with some blue iodide of starch. The solution will also be less opaque, owing to formation of amidulin before the later products make their appearance. At frequent intervals take out drops from test-tube in water bath, and test with iodine. Erythro-dextrin will disappear, as shown by the absence of the reddish tint, achroö-dextrin, the next body to appear, giving no coloured compound with iodine (the achromic point). The action of ptyalin is accelerated by the presence of 0.02 to 2 per cent. of sodium chloride (Cole). If the attainment of the "achromic point" is delayed, add a few drops of 5 per cent. sodium chloride to the digest.

(c) After the tube has remained in the water bath about five to ten minutes, take it out, make mixture alkaline with KOH, add Fehling's solution, and boil; red flakes of Cu_2O separate out if maltose has formed.

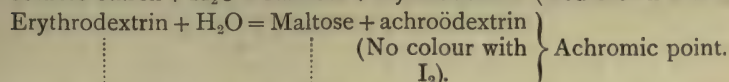
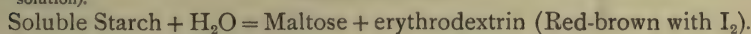
A large number of intermediate substances are formed during the conversion of starch into maltose by the hydrolytic action of ptyalin; and the exact nature of these

derivatives is still uncertain. The following scheme, although not strictly accurate, may serve to render the nature of the process somewhat clearer :—

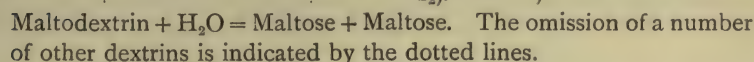


(Opalescent solution).

(Transparent solution).



(No colour with I_2).



The conversion of the starch into maltose is never complete owing to the fact that its rate is reduced by the products of digestion. If the readily diffusible products of digestion, specially maltose, be removed by dialysis the process of hydrolysis is accelerated, and rendered more nearly complete (Lea).

[(d) Test effect of addition of dilute acetic acid and hydrochloric acid solutions on action of saliva.]

Carry out tests as described under (b), only to one test-tube add a few drops of .25 per cent. HCl, to another a few drops of .5 per cent. acetic acid. Note whether action of ptyalin is increased or diminished (hastened or delayed).

(e) Boil saliva and then test its action on starch—nil—owing to destruction of the ferment.

CHAPTER VII

GASTRIC JUICE AND PRODUCTS OF DIGESTION

GASTRIC CONTENTS

THERE are three classes of constituents which require to be recognised—the ACIDS, the unorganised FERMENTS or ENZYMES, and the PRODUCTS of their combined action.

I. THE ACIDS.

The two which are most commonly met with are, HYDROCHLORIC and LACTIC. The tests which are employed for their detection are mainly colour ones, and, as the character of the reaction is much influenced by the presence of digestion products, it is advisable to adopt the following plan or one somewhat similar.

Take six test-tubes and label them alphabetically. Place them in line in a test-tube stand with a sheet of white paper behind.

The different colour indicators may be tested in the following way :—

i. Methyl-violet.

(a) *Into the first test-tube put HCl of the strength that occurs in the gastric juice (say 8 c.c. conc. HCl to 1 litre of water).*

(b) *Into the second, a similar quantity of weak lactic acid (say 8 c.c. to litre of water).*

(c) *In the third, equal quantities of (a) and (b). It*

is advisable in this case to try also the influence of a smaller quantity of lactic acid along with the HCl.

[(d) In the fourth, equal quantities of (a) and a weak proteose solution (e.g. 2 per cent. Witte's peptone in 5 per cent. salt solution).]

[(e) In the fifth, (b) and the same quantity of the weak proteose solution.]

[(f) In the sixth, equal quantities of (a), (b), and the dilute proteose solution.]

Now into each put a drop or two of .1 per cent. methyl-violet and mix.

In (a) the violet colour is changed to blue.

In (b) it becomes bluish-violet.

In (c) the colour change is the same as in (a).

The colour changes for different values of P_H ¹ are green-blue for $P_H=1$, blue for 2, violet for 3

Range 0.1 to 3.2 (Sørensen).

2. Tropæolin OO (.05 to .1 per cent.).

Repeat the experiment, substituting tropæolin for methyl-violet, and again note colour change in each case.

With HCl the colour is crimson, with lactic acid the colour of the indicator is deepened to an orange red.

$P_H=$	1	2	3
Colour	pink	flesh colour	yellow

Range = 1.4 to 2.6 (Sørensen).

3. Congo-red.

Papers soaked in this stain may be used.

The paper on being dipped into the weak HCl solution shows a blue stain, and a more or less similar colour change is seen on dipping the paper into the lactic acid solution.

$P_H=$	3	4	5
Colour	blue	violet	scarlet

¹ The significance of the symbol P_H will be explained in a subsequent chapter.

Place the papers that have been dipped into the HCl and lactic acid solutions into separate test-tubes, and pour over them some ether. On shaking the tubes, the blue stain produced by the latter acid is removed, while that produced by the former remains.

This may be done before adding either of the colour indicators just mentioned.

Tropæolin OO is the most satisfactory of these three indicators, since its range agrees most closely with the reaction of the normal gastric contents ($P_H = 1.77$), and congo-red is the least suitable indicator for testing the reaction of the gastric contents.

4. Günzburg's reagent.¹

This reaction is best shown when process is carried out as follows:—

Take about ten drops of fluid (a), add two or three drops of the freshly prepared reagent, and carefully evaporate to dryness over a small open flame, or preferably on the water bath, blowing upon the mixture and oscillating the dish slightly so as to prevent charring.

The residue acquires a crimson tint. Repeat this reaction with lactic acid. There is no such colour reaction as with the inorganic acid. The residue has only a yellowish-brown colour.

This colour reaction is the most reliable and delicate, giving a positive result with hydrochloric acid up to a dilution of about .0015 per cent. and a negative result even with 5 per cent. of lactic acid; while the other colour indicators give marked colour changes even with dilute lactic acid. Christiansen has proved that titration of the gastric contents gives, with this indicator, results closely agreeing with the true hydrion concentration.

Note down the results in each case.

The two reactions 6 and 7 are mainly employed for the detection of LACTIC ACID.

¹ Phloroglucin, 2 grms. ; vanillin, 1 grm. ; absolute alcohol, 30 grms.

5. Boas' test.¹

Boas' test is carried out in the same way as that of Günzburg. If free hydrochloric acid be present a red residue is left.

6. Uffelmann's reagent.²

Add five or ten drops of this reagent to the weak HCl solution, and shake. Repeat this test with the solution of lactic acid.

Note the decolorisation with solutions containing HCl, and the yellow tint with those containing lactic acid. Observe carefully how the presence of the one interferes with the action of the other.

7. Dilute ferric chloride.

The colour produced when lactic acid solution is acted upon by Uffelmann's reagent is due to the ferric lactate formed. This is seen in the following test :—

To some lactic acid solution add about ten drops of a very dilute ferric chloride solution. The slightly yellow tint of the ferric chloride is changed into a much deeper yellow (ferric lactate). With the dilute HCl the ferric chloride tint becomes slightly less from the dilution.

Before proceeding to methods of separation of the acids, it is advisable to collect the results obtained with all the colour indicators, and arrange them in order of delicacy.

[SEPARATION OF THE ACIDS FROM ONE ANOTHER.]

This is especially desirable when the acids require to be detected in a mixture of gastric contents. They may be separated in either of two ways.

(a) Take equal quantities of the weak hydrochloric and lactic acid solutions in a test-tube (better in a separating funnel, using larger quantities), add rather more than

¹ Boas' reagent is prepared by dissolving 5 grms. of resorcinol and 3 grms. of cane sugar in 100 c.c. of 95 per cent. alcohol.

² To 100 c.c. of 2 per cent. carbolic acid add ten drops of dilute ferric chloride solution.

an equal quantity of ether, and shake thoroughly. Repeat this with fresh quantities of ether two or three times, pouring off ethereal solution each time into another test-tube, and place this in hot water bath until ether has evaporated (with larger quantities distil). Dissolve residue in water, and test with ferric chloride or Uffelmann's reagent.

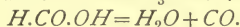
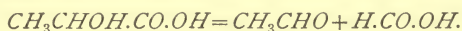
Also test the deeper lying aqueous extract for HCl in the usual way.

The ether dissolves the lactic, leaving the hydrochloric acid mainly in the aqueous solution.

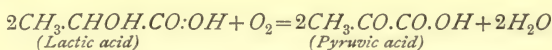
(b) In many cases it is preferable to drive off the water from the mixture of gastric contents, and extract the residue with ether thoroughly in Soxhlet apparatus, the ethereal extract being treated in the usual way by distilling off the ether and testing the residue.

[8. Hopkins' reaction for lactic acid.] Place three drops of a 1 per cent. alcoholic solution of lactic acid in a dry test-tube, add 5 c.c. of concentrated sulphuric acid (free from nitrogen) and three drops of a saturated solution of copper sulphate. Mix and heat the tube in a water bath of boiling water for about five minutes. Cool thoroughly, add two drops of a 0.2 per cent. alcoholic solution of thiophene, and shake. On replacing the tube in the water bath, a cherry-red colour gradually develops.

Note.—Sulphuric acid decomposes lactic acid into acetaldehyde and formic acid. The latter is then decomposed by the concentrated sulphuric acid into water and carbon monoxide.

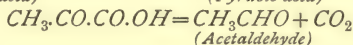


The copper sulphate facilitates the oxidising action of the concentrated sulphuric acid, and therefore part of the lactic acid is probably oxidised to pyruvic acid, which is then decomposed by the concentrated sulphuric acid into acetaldehyde and carbon dioxide, thus:—



(Lactic acid)

(Pyruvic acid)



(Acetaldehyde)

The colour reaction is due to the formation of a compound of acetaldehyde with thiophene (Fearon). The colour is discharged by the addition of traces of water. Hopkins' test may be applied to the dry residue of the ethereal extract, after the latter has been dissolved in alcohol.

II. THE FERMENTS.

They are two in number, PEPSIN and RENNIN, the former acting best in acid, the latter in neutral solution.

The normal reaction of the human gastric contents after a light test meal corresponds to a $P_H = 1.77$. The P_H may rise nearly to 1 in cases of marked hyperacidity (Michaelis, Sørensen, and others).

I. Pepsin.

May be prepared from the fundus mucosa of the pig's stomach, or a commercial preparation may be employed either in powder form or in fluid (liquor pepticus).

If the former, the mucosa should, in the first place, be washed with warm (37°) normal saline, then separated, and the minced mucosa extracted in bottle-shaker with .25 per cent. HCl (8 c.c. HCl to litre) for twenty-four hours. The mixture is first run through muslin and finally through folded filter paper. It is often advisable, instead of using this filtrate as the digesting fluid, to precipitate with alcohol and dissolve the precipitate in .25 per cent. HCl.

If a commercial powder be employed, it is advisable to wash the preparation with water (should it be insoluble in water), and then finally digest it with .25 per cent. HCl at room temperature for a few hours, before using it as a digesting fluid. The extraction is hastened by using a bottle-shaker. About 1 grm. pepsin should be taken for each litre of dilute acid.

Very often also a glycerine extract of the minced mucosa is employed, this being added to the necessary amount of .25 per cent. HCl solution.

A HCl extract of the scrapings obtained from the fundus mucosa of the calf is very suitable for demonstrating both the action of pepsin and rennin.

The activity of these extracts may be tested in different ways. Usually either fibrin or coagulated egg white (in capillary tubes or, better, as fine shavings of coagulated ovalbumin) is taken.

If fibrin be employed (and it is much the best for class purposes), it should in the first place be thoroughly freed from blood pigment, etc., by washing, then put into a basin of slightly acidulated boiling water for a minute or two, cooled, and the water squeezed out. It may be preserved for further experiments in chloroform water or glycerin. It is not necessary to use fibrin stained with neutral carmine.

METHOD OF TESTING FERMENT ACTIVITY.

The experiments should be performed in such a way as to aid one in arriving at a definite knowledge as to the rôle of the acid in different strengths, the influence of variations in temperature, of accumulation of digested products, etc. Such experiments are, however, difficult to carry out properly in a class, and so the following plan may be adopted:—

Take four labelled test-tubes, into each place a strand of washed fibrin. To one add a neutral extract of pepsin, to another .25 per cent. HCl, to another .25 HCl and pepsin solution, and to another pepsin-HCl that has been previously boiled and cooled. Keep all at a temperature of 37° for at least an hour. A few drops of toluene may be added to each test-tube to prevent bacterial action.

The digestion can be carried out in a water bath, the fluid in the tubes being kept at a temperature of about 37°. The tubes may be kept in position by placing them in tin pannikins which have a wire cover, with suitable openings for the tubes. These pannikins may supply the place of water baths, care being taken to employ a small flame. The temperature may be tested with the finger.

It is best at the outset to have a pannikin on each bench with water at the proper temperature, so that the students may observe what is required.

The tubes must be shaken every few minutes, and any changes taking place in the fluid noted down. When solution

of the fibrin in any of the tubes has occurred, the contents should be examined for the products of digestion in the way to be described.

If time permits, a comparison series of tubes with coagulated egg white should be examined ; but perhaps it is better to simply show in a similar series of tubes how much slower digestion goes on in these than in the former series.

These experiments should permit conclusions being drawn as to action of pepsin in neutral and acid solution, and also should show the inactivity of pepsin after boiling. The very slow solvent power of weak HCl will also be shown.

The fibrin in the first test-tube is unaltered, that in the second test-tube swells up and becomes transparent, but does not dissolve unless the acid be allowed to act for several hours. The fibrin in the third test-tube swells up, becomes transparent, and then gradually dissolves. The fibrin in the fourth test-tube undergoes the same changes as that in the second.

Tabulate the results with regard to the time of commencement and the nature of the solvent process in each case.

As the quantities of the digestion products are so small in these test-tubes, owing partly to the small amounts of fibrin and also to the comparatively short time at the disposal of the student, it is advisable to carry out the examination of the products in digested mixtures, previously prepared ; but the fluids in the tubes may be examined in the same way, and the results noted down (for method, *see* "III. Products of Peptic Digestion").

2. Rennin.

Neutralise with weak Na_2CO_3 the acid extract of the calf's gastric mucosa, and test its activity in the following way. A solution of rennet or rennin powder may also be employed, as these usually are very active preparations.

Take four test-tubes, and into each pour some milk. Place in water bath at 40° for a minute or two, then—

(a) To one, add a few drops of a neutral rennin preparation.

(b) *To another, about five drops of .2 per cent. potassium oxalate; shake, and then add a few drops of the rennin extract.*

(c) *To another, add the same quantity of oxalate, and, in addition, one or two drops of 1 per cent. CaCl_2 and a few drops of the extract.*

(d) *To another, add a few drops of a portion of the rennin extract that has been previously heated to boiling point (better boil for about one minute).*

(e) *To another add about five drops of 0.2 per cent. potassium oxalate, shake well, and add a few drops of the rennin extract. Leave on the water bath for half an hour, then boil the fluid to destroy the enzyme and add a few drops of 1 per cent. calcium chloride. The milk clots.*

The results obtained from this series will show—

(a) Action of rennin on ordinary milk.

(b) Action of rennin when the soluble lime salts are precipitated in insoluble form.

(c) The influence of the addition of a small excess of soluble lime salt to oxalate milk.

(d) The effect of boiling upon the activity.

Note the results in each case. Within half an hour the milk clots in experiment (a) fails to clot in (b) because the soluble lime salts have been removed, clots in (c) because sufficient excess of a soluble lime salt has been added, and does not clot in (d) because the enzyme has been destroyed by boiling. The result of (e) indicates that the enzyme produces some chemical alteration of the milk, even when soluble lime salts are absent, but that the latter are essential for the separation of the clot.

The following may be shown, in order to emphasise the difference between coagulation by rennin and coagulation by the fibrin ferment :—

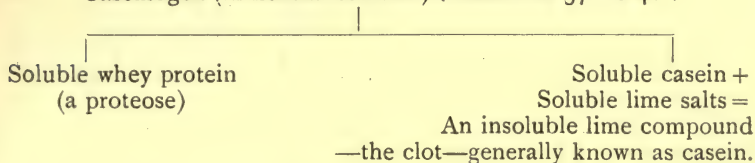
[Dissolve some freshly precipitated caseinogen in very weak alkaline solution. The solution should react neutral. Test to see whether it be free from soluble lime salts; if so, add to some of this solution, in a test-tube, a small

quantity of the rennin solution which has also been rendered free from soluble lime salts. Shake gently, and allow mixture to stand in water bath at 37° for about five minutes. No coagulation ought to occur. Now, bring the solution to boiling point, to destroy the enzyme, and then, after cooling slightly, add a few drops of CaCl_2 , when the separation of an insoluble clot ought to take place.]

That is to say, although the enzyme rennin is not able in Ca-free solutions to separate out from milk an insoluble clot, it is able, under these conditions, so to alter the caseinogen that even after the enzyme has been destroyed by boiling, the mere addition of a soluble lime salt causes the coagulation to take place.

The following scheme indicates the changes which take place in the clotting of caseinogen :—

Caseinogen (in neutral solution) + rennin at 37° to 40° .



III. PRODUCTS OF PEPTIC DIGESTION.

As previously mentioned, the contents of the tubes used in the earlier digestion experiments may be employed for this separation ; but it is better to use the following :—

1. A 2 per cent. solution of Witte's peptone¹ in 1 per cent. NaCl solution.
2. A peptic digest of three hours' duration.
3. A peptic digest of twenty-four to forty-eight hours' duration.

In both of the last-mentioned cases fibrin should be used.

METHOD OF DETECTION AND SEPARATION.

It is advisable to work with fairly large quantities, say 25 to 50 c.c. Therefore, take in a small flask, about this

¹ Other forms of commercial peptone may replace Witte's.

quantity of the digested fluid (obtained by filtering off the undissolved material), add a few drops of litmus solution, and then, drop by drop, very dilute NaOH until reaction is almost neutral, but still remains slightly acid (note, in fact, when maximal precipitation occurs). Filter off any precipitate = acid albumin (this may be dissolved in weak alkali, and examined, as described on pp. 70, 71). Bring the neutral filtrate almost to boiling point, then acidify slightly with acetic acid; boil and remove any precipitate of coagulable proteid by filtration. Now, measure volume of filtrate, and add an equal quantity of neutral saturated ammonium sulphate solution, shake thoroughly, and filter. The precipitate is one of primary proteoses (proto- and hetero-proteose¹). The filtrate may contain secondary albumoses and peptone. The precipitate (a) and the filtrate (b) are to be treated separately. Wash the precipitate (a) with half-sat. $(\text{NH}_4)_2\text{SO}_4$, and then dissolve it in as little water as possible. The methods employed for separation of these primary albumoses will be given afterwards.

The filtrate (b) is treated in the following way:—Powder some $(\text{NH}_4)_2\text{SO}_4$ in a mortar (rather more than requisite to completely saturate the filtrate), warm the filtrate to about 60° , and pour it upon the powdered $(\text{NH}_4)_2\text{SO}_4$. Acidify with dilute H_2SO_4 that has been saturated with $(\text{NH}_4)_2\text{SO}_4$, mix up thoroughly, and filter. The precipitate so obtained is one of secondary proteoses (deutero-proteoses), and is, in the case of peptic digestion, small in amount.

The filtrate may contain pure peptone (usually only a very small quantity, unless in the case of prolonged digestion). Dissolve the precipitate of secondary proteoses in water, and test as described on p. 70.

[Evaporate the filtrate containing peptone (?) to about

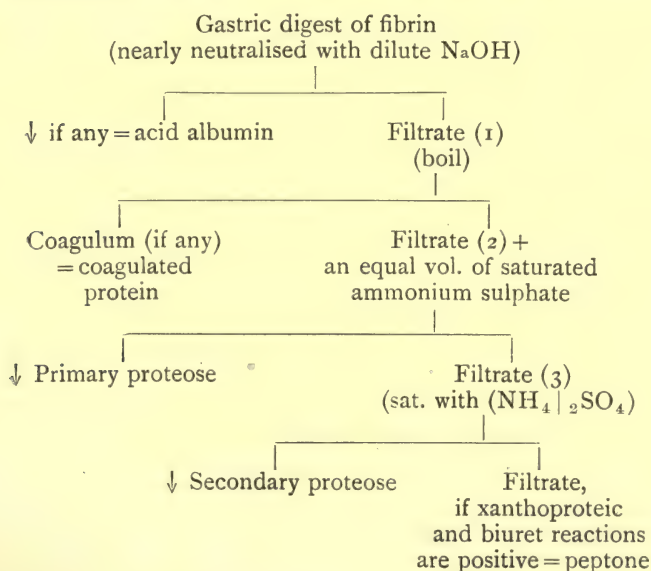
¹ Another primary proteose is sometimes referred to, namely, dysproteose. It is, however, only an insoluble modification of hetero-proteose.

one-quarter of its volume, cool, and remove the $(\text{NH}_4)_2\text{SO}_4$ that separates out. The remainder of the $(\text{NH}_4)_2\text{SO}_4$ may be removed by adding a saturated solution of $\text{Ba}(\text{OH})_2$ until all the sulphate is precipitated as BaSO_4 . Filter.]

The presence of peptone in the filtrate, if all the $(\text{NH}_4)_2\text{SO}_4$ has not been removed, may be shown by adding a larger quantity of KOH than is required to set free all the NH_3 from the ammonium salt, warming (to drive off the ammonia), and then adding a drop or two of very dilute CuSO_4 . A pink reaction, which may be masked somewhat by the ammonium cupric hydrate, signifies the presence of true peptone. It is absolutely essential to add a large excess of KOH if there be much $(\text{NH}_4)_2\text{SO}_4$ present.

It is simpler to test the filtrate (after removal of the proteose) by the xanthoproteic reaction.

The method of separation may be summarised as follows:—



[METHOD OF SEPARATING PRIMARY PROTEOSES FROM ONE ANOTHER.]

(a) *To about 3 c.c. of the solution of primary proteoses add an equal quantity of methylated spirit. Hetero-albumose is precipitated, while proto-proteose is not, the latter being very soluble in all alcohols up to about 80 per cent.; the former, on the other hand, being precipitated even by 32 per cent. alcohol.*

(b) The ordinary method that is employed is more tedious. It depends upon the fact that proto-proteose is soluble in distilled water, while hetero-proteose is not.

[Fill a sausage tube (Kühne's)¹ with some of the primary proteose solution. Pass a glass tube through the ends, and dip the loop into a vessel containing water. Circulate water around the loop by connecting water tap with rubber tubing which dips into glass vessel. After some hours, pour out a little of the mixture, and see if there be suspended material. If not, circulate water for some time longer, then slit open tube, collecting the fluid, and see if there be any albumose adhering to the paper. This precipitate = hetero-proteose. The filtrate may contain proto-proteose, which will give the ordinary proteose reactions.]

The hetero-proteose may be dissolved in weak salt solution and tested with the usual proteose reagents.

Instead of separating the primary proteoses by half saturation with $(\text{NH}_4)_2\text{SO}_4$, one may adopt the usual method that is described, namely, complete saturation with NaCl in neutral solution. On acidification, however, the secondary proteoses are not completely precipitated, while, if the NaCl solution be neutral, the proto-proteose is also not completely separated. For these reasons this method is not so satisfactory as the one which is described above.

¹ The parchment tube should be washed before use in several changes of distilled water to remove adherent salts and other foreign material, and tested for leaks. The latter can frequently be sealed up with egg white, which is afterwards coagulated by heat. Collodion sacs may be used with advantage instead of parchment paper tubes.

If the solution containing true peptone be freed from $(\text{NH}_4)_2\text{SO}_4$ completely, then, after evaporating the fluid down to small bulk, the peptone may be precipitated by absolute alcohol, and should then, after careful washing with alcohol, be preserved in an air-tight bottle.

State in tabular form the results of your examination. It is very advisable to carry out the complete method just described, in the case of mixtures obtained after varying periods of digestion ; for example, the three referred to on p. 113.

It is very instructive also to study the products derivable from peptic digestion of milk that has been clotted with rennin. The undissolved residue, the so-called pseudo- or para-nuclein,¹ may be incinerated and tested for phosphorus, as described on p. 6.

Some of the most instructive reactions given by these digestion products depend upon the presence of particular groups, *e.g.* the primary proteoses do not give the furfural reaction, while the secondary do,² and both the primary and secondary (at least the mixture of the latter) give the sulphur tests, while peptone does not.

This method of differentiating these products has been carried out to a large extent within recent years.

¹ It is better to term this residue the phosphorus-holding residue remaining after gastric digestion of a phospho-protein, such as caseinogen or vitellin.

² Hopkins and Cole (*Proc. Royal Society*, lxviii. p. 32) have thrown doubt upon this statement made by Pick (*Zeit. f. physiol. Chem.*, xxviii. p. 219).

CHAPTER VIII

PANCREATIC AND INTESTINAL DIGESTION

IN the pancreatic juice four enzymes may be found—a proteolytic one, *trypsin*; an amylolytic one, *amylopsin*; a fat-splitting, *steapsin*; and a milk-curdling, *rennin*. It is often difficult to say whether the last-mentioned is present or not, as, in active proteolytic extracts, the trypsin may mask the curdling, owing to the solution of the clot.

Digestion experiments may be performed with the pure pancreatic juice after its zymogens have been transformed into active enzymes by action of the succus entericus (enterokinase) or by addition of active tryptic extract. As a rule, however, extracts of the pancreas are made, and from these more or less pure ferment preparations may be separated. Very frequently commercial preparations, such as the liquor pancreaticus (Benger), or pancreaticum siccum, are employed, but a very active extract may be obtained in the following way:—

Take a fresh pig's pancreas, from which as much of the fat has been removed as possible, mince up finely, leave exposed in a thin layer for two to six hours at room temperature. Then add, for every gramme of tissue, about 4 c.c. of 50 per cent. glycerol, very slightly acidified with acetic acid, a little toluene being added to prevent bacterial growth. Shake up thoroughly in bottle-shaker for three or four hours, and allow mixture to stand twenty-four hours, or longer. Pour off fluid, and filter if necessary.

Before using this extract, dilute it with an equal quantity of water. It may be employed for experiments on the proteolytic, diastatic, and rennetic properties of the pancreatic juice.

Other extracts—weak alcoholic, saline, aqueous (with

chloroform or toluene added)—are often employed. A most active proteolytic extract may be obtained by extracting the minced gland for twenty-four hours with water rendered slightly alkaline with ammonia, filtering and precipitating the nucleo-protein with dilute acetic acid. This precipitate may be dissolved in .2 to .8 per cent. Na_2CO_3 , and forms a most suitable digesting fluid.

A. TRYPSIN.

I. METHOD OF TESTING PROTEOLYTIC ACTIVITY.

It is absolutely necessary, when dealing with pancreatic, preparations, to test their activity beforehand. Thus, with glycerine extracts, the activity will largely depend upon the duration of extraction. If a dilute glycerine be used, then the extract is suitable for digestion experiments much sooner than if a stronger glycerine be employed. The activity seems often largely to depend upon the condition of the gland before extraction as to whether it has been exposed to air or not.

The addition of a saline extract of the duodenal mucosa may increase the ferment activity, the enterokinase setting free active enzyme from inactive zymogen.

Fibrin is, on the whole, the best protein for digestion experiments in a class.

The fresh fibrin should be passed through boiling water before subjecting it to digestion.

Sufficient fibrin for the class should be digested for about an hour with .5 per cent. Na_2CO_3 in the incubator.

The experiments should be of such a nature as to demonstrate the influence of reaction and temperature upon the digestive activity of the ferment.

Take four test-tubes, into two of these pour some .2 to .5 per cent. Na_2CO_3 , add five to ten drops of the active extract to one, and five to ten drops of the previously boiled and cooled extract to the other. Into a third test-tube pour the Na_2CO_3 solution, without addition of extract; and into the fourth, a weak HCl solution (.2 per cent.) and a few drops of the extract.

Into each, place approximately the same quantity of fibrin (small amount), treated as above described, and place the four tubes in water kept at 38°. Every five or ten minutes shake the tubes; so as to mix the digestive products and permit of more favourable digesting conditions.

The tubes should be left in the water bath for one to two hours until most of the fibrin has been digested. The plan adopted for the examination of the contents of the tubes is practically the same as for separation of peptic products, and may shortly be given, certain precautions, etc., being omitted, as they are referred to later on. Note that the fibrin in the first tube has gradually undergone disintegration and dissolved without swelling up, that the fibrin in tubes 2 and 3 has undergone no change, and that the fibrin in tube 4 has swollen up but not dissolved.

The contents of each tube are to be treated in the same way.

(a) *Filter contents of tube, add litmus, neutralise with very dilute acetic acid; if precipitate (rarely occurs) = alkali albumin.*

(b) *Filter, boil; if coagulum = unaltered coagulable protein.*

(c) *Filter, saturate with $(\text{NH}_4)_2\text{SO}_4$, acidify with dilute H_2SO_4 (saturated with $(\text{NH}_4)_2\text{SO}_4$), thoroughly mix; precipitate = secondary albumoses.*

(N.B.—There are no primary albumoses, or only traces.)

(d) *Filter. Divide the filtrate into two portions. To one add excess of KOH and a drop of dilute CuSO_4 ; if pink = peptone.*

Test the other portion for the xanthoproteic reaction.

II. SEPARATION OF PRODUCTS OF TRYPTIC DIGESTION.

Place two handfuls of freshly washed fibrin in a 2-litre flask, add about 1 litre of .5 per cent. Na_2CO_3 , and 5 c.c. toluol, leave in incubator at 37° about two hours, then add about 5 grms. of minced pancreas that has been exposed to the air

for about twelve hours. Before adding the pancreas to the alkaline solution, it is advisable to treat it in the following way:—Mix up with absolute alcohol in mortar, then replace alcohol by ether. The ether is then squeezed out, and the mass spread in thin layer on filter paper to remove traces of ether.

Shake the flask every hour or so, and, after about six hours, filter off a portion for examination (PORTION A). Replace flask in incubator and leave for three days. Small quantities of fresh pancreas powder (or other preparation) should be added each day, as a large proportion of the ferment is destroyed by the alkali. Filter, and divide the fluid into two portions (B and C), one (B) to be employed for examination of digestion products of protein nature, the other (C) for leucine and tyrosine.

PORTIONS A and B are to be treated in the same way, and results noted down. About 25 to 50 c.c. in a flask are given to each student.

(a) *Add a few drops of litmus to the solutions, then neutralise with dilute acetic acid (or rather add dilute acetic acid very carefully until neutral point is reached, then add a trace more, noting when maximal precipitation occurs); any precipitate = alkali albumin.*

(b) *Filter, bring filtrate to boiling point, acidify slightly with dilute acetic acid, precipitate = coagulable protein.*

(c) *Filter, pour a small quantity of each filtrate into test-tube, and add a few drops of bromine water. Violet colour signifies presence of tryptophane.*

(d) *Add an equal quantity of saturated ammonium sulphate to the rest of the filtrate: If any precipitate (?) = primary proteoses.*

(e) *Filter, if necessary. Warm filtrate to about 50° to 60°, and pour it upon powdered $(\text{NH}_4)_2\text{SO}_4$, sufficient to saturate the solution. Shake thoroughly, acidify with dilute H_2SO_4 (saturated with $(\text{NH}_4)_2\text{SO}_4$); precipitate = secondary proteoses (deutero-protease).*

(f) *Filter, evaporate down to about one-quarter of its bulk. Cool, filter off excess of $(\text{NH}_4)_2\text{SO}_4$, and test filtrate (either directly or after removal of the rest of the $(\text{NH}_4)_2\text{SO}_4$ by $\text{Ba}(\text{OH})_2$) by Piotrowski's test (the usual precaution being taken of adding a large excess of KOH before adding the drop of dilute CuSO_4 , see p. 70. If pink colour appears, then peptone is present.*

The presence of peptone in the filtrate after removal of the secondary proteoses may be shown by the xanthoproteic reaction being given. A positive xanthoproteic reaction, however, may be due to simpler decomposition products of protein, such as tyrosine. Tabulate the results obtained by the examination of the two fluids (six and seventy-two hours' digestion).

PORTION C (FOR LEUCINE AND TYROSINE).

METHOD OF SEPARATION.—Evaporate down the fluid (if necessary, freed from any coagulable or alkali albumin) which has been acidified with acetic acid until it just begins to become syrupy. Leave overnight in a cool place and examine the crystals microscopically. The first crop is, as a rule, mainly composed of tyrosine, especially if the solution be not very concentrated.¹

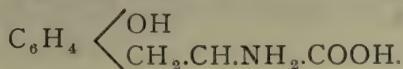
Pour off the fluid, running it through fine muslin, and evaporate filtrate down to thick syrup. Leave overnight. The second crop of crystals, which forms a skin on the surface of the thick syrup, is mainly composed of leucine, or contains a larger quantity of leucine than the first crop. Examine crystals microscopically, and sketch both forms. The fluid from which the crystals have separated should be examined for peptone.

¹ The crystals of leucine and tyrosine may be shown, even although the students do not separate those bodies from the digested mixture.

Leucine crystallises in the form of round balls made up of concentrically arranged lighter and darker areas, and showing radial striations.

Tyrosine crystals are fine colourless needles often arranged in clusters (see Plate at end of Book).

Tyrosine (p. oxyphenyl α -aminopropionic acid).



1. *Examine the crystals and sketch them. Warm the slide slightly. They do not melt at a temperature when crystals of somewhat similar appearance (fatty acids and fats) do.*

2. *Add some of the crystals to a little water in a test-tube. Then add a few drops of Millon's reagent, and heat. Fluid becomes red in colour, and a reddish-brown precipitate appears later (see p. 70).*

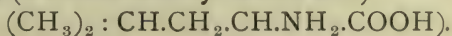
[3. **PIRIA'S TEST.**—Take a small quantity in a dry test-tube; pour over it a small quantity of pure conc. H_2SO_4 . Shake. Keep immersed in water bath for half an hour. Cool. Empty into a beaker containing five times its quantity of distilled water; add dry BaCO_3 until neutral reaction, stirring constantly. Filter, evaporate filtrate down to small bulk, and add very carefully one or two drops of dilute ferric chloride; solution becomes violet in colour. This reaction is due to the formation of tyrosine-sulphonic acid.]

[4. *Dissolve a small quantity in hot water to which a crystal of NaCl has been added, and then add a small quantity of dry quinone. The warm solution becomes ruby-red in colour.*]

[5. *Add 2 c.c. of a formaldehyde-sulphuric acid solution (1 c.c. formaldehyde in 50 c.c. conc. H_2SO_4) to a small quantity in a test-tube. Whenever the solution becomes red in colour, add double its volume of glacial acetic acid, and boil. The colour then changes to green.*]

[6. **SCHERER'S TEST.**—(Carry out as described under Leucine, 4.) The formation of the yellow nitrate of nitroso-tyrosine is characteristic. On dissolving this residue in NaOH , the colour becomes orange.]

Leucine (α -amino-isobutyl acetic acid).



1. *Examine microscopically a drop taken from the crystalline surface layer of the thick syrup. Sketch the crystals (cf. form of purer crystals separated out from a hot ammoniacal alcoholic solution).*

[2. *Dissolve some purified crystals in KOH, and carefully add dilute CuSO_4 , shaking after addition of each drop. The $\text{Cu}(\text{OH})_2$ is dissolved, giving a light blue-coloured solution. Heat, no reduction.*]

[3. *Heat carefully some leucine between two cover-glasses; a fine white sublimate is formed on the upper glass. Note the peculiar odour of amylamin.*]

[4. **SCHERER'S TEST.** — *Take a small quantity of leucine on platinum foil, add a drop or two of concentrated nitric acid, and heat carefully. A colourless residue is left, which can be dissolved in warm NaOH, the resultant fluid being colourless. This fluid, on being evaporated down, leaves an oily residue.*

Do the same with tyrosine and note the difference at each stage (see Test 6, Tyrosine).]

[5. *When free from tyrosine, leucine does not give Millon's reaction.*]

B. THE AMYLOLYTIC FERMENT OR ENZYME (AMYLOPSIN OR AMYLASE).

A suitable preparation for demonstrating the activity of this ferment is the 50 per cent. glycerol extract of the pig's pancreas, prepared as described on p. 118; or a strong watery extract of pancreas powder may be made by digesting about 2 grms. with 100 c.c. of toluol water, at 38° , for about two hours.¹

Take four test-tubes, and place in them glass rods. Pour into all 1 per cent. starch solution; into one of these, pour five to ten drops of the extract (if the glycerol one be used, dilute it with equal quantity of water before adding). Add five to six drops of a previously boiled and

¹ Any other pancreatic preparation may be employed.

cooled extract to the starch in the second test-tube. Into a third put a few drops of bile; and into the fourth, extract and bile. In this way one may test activity of extract with and without bile, and after it has been boiled. Place all in water bath. On a porcelain slab at the side, place a series of iodine drops. Examine in same way as with saliva. Note the "achromic" point, when there is no reaction to iodine. The action of the amylopsin is greatly accelerated by the addition of sodium chloride, the optimal concentration being .016 N. (Mellanby and Wooley.) Place five to six c.c. of the starch solution in a fifth test-tube, add ten drops of the extract, and about 1 c.c. of N/10 sodium chloride. Note that the time required to attain the achromic point ("the achromic period") is reduced.

Also test, in conclusion, with Fehling, for maltose.

The changes are the same as with saliva (*see* p. 103).

It is advisable in this, as in the case of trypsin, to try the effects of various extracts.

The effect of bile upon the activity of the extract is very doubtful. The boiled extract is inactive.

C. THE FAT-SPLITTING ENZYME.

Take some butter which has been freed from fatty acids in the following way:—Dissolve some butter in ether. Pour into separating funnel, and then add a small quantity of dilute NaOH. Shake thoroughly. Run out the aqueous alkaline solution, replace with fresh NaOH, shake, and again remove it. Remove all traces of alkali from ethereal solution by shaking up repeatedly with small quantities of water. Run ethereal solution into flask containing anhydrous CaCl_2 , shake, and leave overnight. Next morning pour off ethereal solution into distilling flask, and distil off the ether. Melt the purified butter, now freed from fatty acids, and run it into a sterilised vessel, which may be sealed.

[Take two small, wide-mouthed Soxhlet flasks, add to one some freshly minced pancreas, suspended in water con-

taining a little chloroform; to the other, the same, only previously heated to boiling point. Add a few drops of an alcoholic solution of rosolic acid (which gives a pink with alkalies, yellow with acids) to each, and then just sufficient dilute NaOH to give red colour. Place both in incubator, at about 38°, and at intervals take them out and mix thoroughly by shaking. Note the time when the red colour is discharged by fatty acids, which have been set free. This may take a fairly long time (twelve hours or so).]

If the discharge of colour is due to the splitting of the fats by a ferment, the boiled extract should not show this reaction. The smell of volatile fatty acids is often very distinct. The fatty acids may be separated and tested.

D. THE MILK-CURDLING ENZYME.

Various extracts may be tested for this in the way described for rennin in the gastric juice.

Some test the activity by the rapidity of production of the so-called meta-casein reaction (the appearance of a coagulum in milk on boiling), others by the ordinary insoluble clot formation.

E. ENZYMES OF THE INTESTINE.

The succus entericus contains the enzymes:—erepsin (which hydrolyses proteoses, peptones, and casein, but has no action on most native proteins), maltase, lactase, and invertase (sucrase), which hydrolyse the corresponding disaccharides, enterokinase, which converts trypsinogen into trypsin, and the hormone secretin, which stimulates pancreatic secretion. Other enzymes have also been obtained from the mucous membrane of the small intestine such as nuclease, arginase, pepsin, or pseudopepsin from Brunner's glands, and a lipase.

I. EREPSIN.

Preparation of an intestinal extract containing erepsin, maltase, lactase, and sucrase.

A piece of small intestine is obtained from a pig, the mucous membrane is exposed, washed with water, scraped off, weighed, ground up with sand, and transferred by washing with about 15 times its weight of water into a stoppered bottle, a little toluene being added. 1 grm. of sodium chloride is added for each 100 c.c. of fluid, the mixture is kept for twelve hours at room temperature, and then filtered through well washed calico. The filtrate is used for the following experiments:—

Prepare 200 c.c. of a 2 per cent. solution of commercial peptone in 0.9 per cent. sodium chloride. Divide into two equal portions, and place each in a stoppered bottle, adding some toluene to prevent putrefaction. Add to one (labelled A) 20 c.c. of the solution of erepsin, and to the other (labelled B) 20 c.c. of previously boiled and then cooled erepsin. Leave both bottles in the incubator at 37° to 40° for two or three days.

1. Apply the biuret test to small portions of each solution. It is either negative or faint with (A), but distinct with (B).

2. Acidify small portions of (A) and (B) with dilute acetic acid and test for tryptophane with bromine water. A distinct colour is obtained with (A), no colour change, or at most a faint one with (B). Some samples of commercial peptone contain traces of tryptophane, and therefore give a faint positive reaction.

The action of erepsin on casein may be demonstrated in a similar way.

II. INVERTASE, LACTASE, AND MALTASE.

Place two 20 c.c. portions of 2 per cent. solutions of each of the three sugars in six labelled stoppered flasks. Add 5 c.c. of the intestinal extract to the sucrose, lactose, and maltose solutions. Add 5 c.c. of previously boiled and cooled extract to each of the control set of flasks containing the three sugars. After adding toluene, leave the six flasks in the incubator at 37° to 40° for two to four days.

1. Portions of the solutions from the first series of flasks will be found to reduce Barfoed's reagent, and the solution of cane sugar or sucrose, which has been hydrolysed to glucose and

fructose, will be found to reduce Fehling's and Barfoed's reagents.

2. Portions of the solutions from the second series of flasks will be found not to reduce Barfoed's reagent. The solution of sucrose will also be found to be unaltered, reducing neither Fehling's nor Barfoed's reagents.

CHAPTER IX

BACTERIAL DIGESTION¹

THIS can be best studied in a decomposing alkaline meat extract. As one wishes to examine also for the ordinary proteolytic products, it is better not to use a decomposing pancreatic extract, as then there is the combined action of trypsin and bacteria. Take half a pound of finely minced meat in a large flask, and add about a litre of .5 per cent. Na_2CO_3 . Insert a plug of cotton-wool in the mouth of the flask. Shake thoroughly, and place in incubator, at a temperature of 40° , for a week. It is advisable, at the outset, to introduce a small piece of putrefying meat into the mixture. The mixture has an extremely offensive odour.

I. ALBUMOSES AND PEPTONES.

In one portion test for albumoses and peptones.

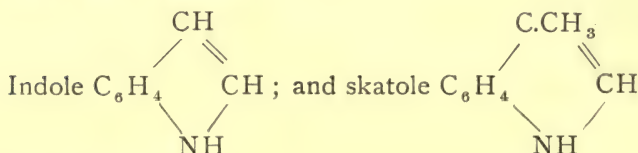
II. INDOLE, SKATOLE, VOLATILE FATTY ACIDS, PHENOL, CRESOL, AND AMMONIA.

From the remainder of the fluid distil the volatile products, collecting the distillate until the contents become so thick that bumping becomes troublesome. Take two samples of the distillate, an early (1) and a late one (2). Test the reaction (ammonia). The skatole (or scatole) comes over earlier than the indole, and so it is advisable to keep those samples separately in order that they may be examined

¹ The work referred to in this chapter should only be carried out in an advanced class.

afterwards for indole and skatole. Remove the flame, and when residue is cool add more water and continue the distillation. After about 500 c.c. in all have distilled over, acidify distillate with HCl, and extract it in small portions of 50 to 80 c.c. with about same quantity of ether in a separating funnel. In shaking this mixture, be careful to allow any gases to escape. Repeat this with fresh portions of the distillate and fresh ether. Collect all the ethereal extracts, having run away the acid aqueous ones, which mainly contain NH_4Cl . Extract these with NaOH solution in a separating funnel. The alkaline solution (3) takes up the *phenol*, *cresol*, and *volatile fatty acids*; the ethereal (4) the *skatole* and *indole*. The ether is distilled off from (4), the residue dissolved in as little hot water as possible, and the indole and skatole left to separate out, the former being more soluble than the latter.

Indole and Skatole.



The fractions (1) and (2) and the residue from (4) should be examined.

1. *Examine crystals microscopically. Both crystallise in same form. Warm slide gently. The indole melts at a distinctly lower point than the skatole (52° instead of 95°).*

This is only made out if one is dealing with fairly pure material.

2. In the first place, test the two fractions of the distillate (1) and (2) as follows:—

Take small quantities of each in test-tubes, make acid with a few drops of nitric acid, and add one or two drops of dilute KNO_2 solution (.02 per cent.). If indole be present, even in very small quantity, the solution becomes

red in colour; or, if a larger amount be present, a brick-red precipitate of nitrate of nitroso-indole will separate out. If skatole alone be present, only a white clouding appears. In the second place, test in same way the hot water extract of the ethereal residue from (4), and the insoluble residue left after extraction with hot water.

Note whether indole and skatole are both present.

LEGAL'S TEST.

3. Take some of (1), (2), and of the hot water extract of residue (4) in different test-tubes; add a few drops of freshly prepared sodium nitro-prusside until solution is distinctly yellow in colour. Then add a few drops of NaOH, deep violet colour if indole, intensely yellow if skatole alone be present. Acidify strongly with glacial acetic acid; if indole be present, the solution becomes a bright blue; if skatole, the colour becomes gradually violet on boiling.

[4. EHRLICH'S TEST.] Add to a small quantity of an indole solution about half its volume of a 2 per cent. alcoholic solution of *p*-dimethylaminobenzaldehyde, and drop by drop 25 per cent. hydrochloric acid until a red colour appears. The red colour is darkened by the addition of a few drops of 0.5 per cent. sodium nitrite.

5. Indole solutions give a red colour with Hopkin's and Coles' glyoxylic acid test.

6. KONTÓ'S REACTION.—Add three drops of a 4 per cent. solution of formaldehyde to 1 c.c. of the indole solution previously freed from phenols and ammonia, then add an equal volume of concentrated sulphuric acid, and mix the fluids thoroughly. In the presence of indole, the fluid acquires a reddish-violet colour. Skatole gives a yellow or brown colour under the same conditions.

[7. PYRROLE REACTION.] Immerse a pinewood splinter, previously moistened with concentrated hydro-

chloric acid, in an alcoholic solution of indole. The splinter becomes red. This reaction is given by all pyrrole derivatives.

Phenol, C_6H_5OH , and cresol, $p-C_6H_4(CH_3).OH$ in the alkaline washings (3).

Acidify the solution (3) with HCl , and then add Na_2CO_3 until the reaction (after driving off the CO_2) is just alkaline. The fatty acids are now in the form of sodium salts, and the phenol and cresol can be separated from them by extracting with ether in a separating funnel (leaving free exit for gas). The ethereal extract contains the phenol and cresol, while the salts of the fatty acids remain in the watery solution. Evaporate off the ether from the extract; dissolve the residue in hot water, and test for phenol and cresol. It is not necessary to separate these.

1. *Add bromine water gradually to the solution until maximal precipitation. At first only a transient milky clouding, then a yellowish-white precipitate of tribromophenol and -cresol.*

2. *Boil some of the solution to which Millon's reagent has been added. Fluid becomes red, and a similarly coloured precipitate may separate out. This reaction depends upon the OH group in the benzene ring.*

3. *Add two or three drops of dilute ferric chloride to the neutral solution—violet or greyish-blue colour.*

Salts of the fatty acids.

These are present in the watery solution, and may be examined in the usual way (see "Fats").

In conclusion, tabulate the results obtained by analyses of gastric, pancreatic, and bacterial digestion.

CHAPTER X

THE AMINO ACIDS DERIVED FROM PROTEINS

WHEN proteins are more or less completely hydrolysed by boiling with mineral acids or with alkalis, they yield a large number of amino acids. The proteolysis with enzymes is always less complete, polypeptides being found amongst the products even after the successive action of trypsin and erepsin. Certain amino acids, notably tryptophane, are decomposed by the action of acids, and, to a less extent, by that of alkalis. Tryptophane can, therefore, only be obtained as a product of enzyme action. The amino acids obtained by proteolysis with alkalis are largely racemised, or consist of optically inactive mixtures. Those resulting from acid proteolysis are optically active, when they fulfil the condition for optical activity of containing one or more asymmetric carbon atoms. We have already seen that certain amino acids undergo secondary changes as the result of the action of intestinal bacteria.

Amino acids are derivatives of organic acids, in which one or more hydrogen atoms of the latter have been replaced by amine groups. One of the amine groups is almost invariably in the α -position—*i.e.*, attached to the same carbon atom as the carboxyl group. The only exceptions to the latter rule are certain amino acids formed as secondary products of bacterial action on diamino acids. All the amino acids are amphoteric electrolytes or ampholytes forming compounds with acids in which the amino-acid radicle is a kation, and with bases in which the amino-acid radicle is an anion. The basic properties of the amino acid are due to the amine group, while the acid properties are due to the carboxyl group.

The following is a list of the chief amino acids :—

Class I. Monamino-Monocarboxylic Acids of the Aliphatic Series.

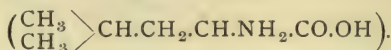
1. **Amino-formic** or carbamic acid ($\text{NH}_2\text{CO.OH}$) is incapable of existing as a free acid, but forms somewhat unstable salts, the best known of

which are ammonium carbamate ($\text{NH}_2\cdot\text{CO}\cdot\text{ONH}_4$) and calcium carbamate. The former salt is known as a precursor of urea.

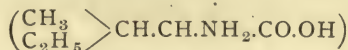
2. **Glycine** or amino-acetic acid ($\text{CH}_2\text{NH}_2\cdot\text{CO}\cdot\text{OH}$).
3. **d-Alanine** or α -amino-propionic acid ($\text{CH}_3\cdot\text{CH}\cdot\text{NH}_2\cdot\text{CO}\cdot\text{OH}$).
4. **d-Valine** or α -amino-isovalerianic acid—



5. **l-Leucine** or α -amino-isobutylic acid—

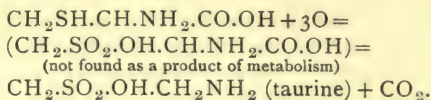


6. **Isoleucine**—



contains two asymmetric carbon atoms. (3) to (6) are optically active.

The amino acids, l-serine and cysteine, are closely related to alanine. Serine ($\text{CH}_2\text{OH}\cdot\text{CH}\cdot\text{NH}_2\cdot\text{CO}\cdot\text{OH}$) is β -hydroxyalanine, and cysteine ($\text{CH}_2\text{SH}\cdot\text{CH}\cdot\text{NH}_2\cdot\text{CO}\cdot\text{OH}$) is the corresponding thio-derivative of alanine. The latter is usually obtained by the reduction of cystine or dicysteine [$\text{S}_2(\text{CH}_2\cdot\text{CH}\cdot\text{NH}_2\cdot\text{CO}\cdot\text{OH})_2$]. In the organism part of the cysteine is oxidised to taurine, and the latter is excreted in the bile as sodium taurocholate.

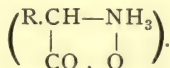


The latter amino acid is not one of the products of acid, alkali, or enzyme proteolysis.

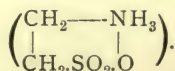
Class II. Aromatic Amino Acids.

1. **l-Phenylalanine** ($\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}\cdot\text{NH}_2\cdot\text{CO}\cdot\text{OH}$).
2. **l-Tyrosine** or p-hydroxy-phenylalanine—
(1.4. $\text{C}_6\text{H}_4\text{OH}\cdot\text{CH}_2\cdot\text{CH}\cdot\text{NH}_2\cdot\text{CO}\cdot\text{OH}$).

All these amino acids are neutral ampholytes, since each contains one amine and one carboxyl group, and these groups tend to unite together to form an internal salt—



Taurine forms a similar internal salt—

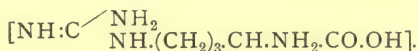


Class III. Monamino-Dicarboxylic Acids of the Aliphatic Series.

1. **Aspartic** or amino-succinic acid ($\text{HO.OC.CH}_2\text{.CH.NH}_2\text{.CO.OH}$).
 2. **Glutamic** or α -amino-glutaric acid [$\text{HO.OC.}(\text{CH}_2)_2\text{.CH.NH}_2\text{.CO.OH}$].
- These are acid ampholytes containing one free carboxyl group. β -hydroxy-glutamic acid has also been recently isolated (Dakin).

Class IV. Diamino-Monocarboxylic Acids of the Aliphatic Series.

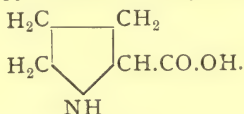
1. **Ornithine** or α - δ -diaminovaleric acid [$\text{NH}_2\text{.}(\text{CH}_2)_3\text{.CH.NH}_2\text{.CO.OH}$].
2. **Lysine** or α - ϵ -diamino-caproic acid [$\text{NH}_2\text{.}(\text{CH}_2)_4\text{.CH.NH}_2\text{.CO.OH}$].
3. **Arginine** or α -amino- δ -guanidovaleric acid—



These are basic ampholytes containing a free amine group.

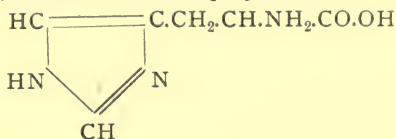
Class V. Heterocyclic Amino Acids.

1. **Proline** or α -pyrrolidine carboxylic acid—

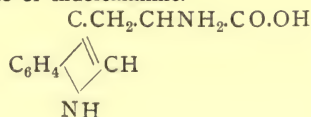


2. **Oxyproline** [$\text{C}_5\text{H}_8(\text{OH})\text{NO}_2$].

3. **Histidine** or β -iminazole- α -amino-propionic acid—



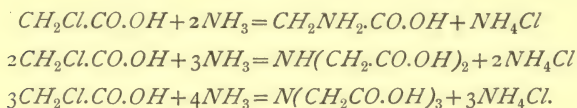
4. **Tryptophane** or indolealanine.



PREPARATION OF SOME AMINO ACIDS.

I. Glycine.

Place 1400 c.c. of concentrated ammonia in a large wide-mouthed bottle, and dilute with 400 c.c. of water. Fit up a mechanical stirrer to be driven by a water turbine or other form of small motor. Dissolve 150 grms. of monochloroacetic acid in 200 c.c. of water, and transfer the solution to a separating funnel. Drop the solution of chloroacetic acid into the ammonia, which is meanwhile continuously stirred. When all the chloroacetic acid has been added, remove the stirrer, stopper the bottle, and allow the solution to stand for 24 hours. Transfer the solution to a flask and partially free it from ammonia by a current of steam, then evaporate in a porcelain basin on the water bath until the odour of ammonia is no longer perceptible. The following reactions take place:—



The first reaction is obviously favoured by large excess of ammonia, gradual addition of chloroacetic acid, and thorough shaking.

Add freshly precipitated copper oxide (prepared by adding caustic soda to a hot solution of 150 grms. of copper sulphate, and washing thrice by decantation) to the warm solution of glycine and ammonium chloride, boil, filter while hot, and evaporate the filtrate on the water bath until crystallisation is found to occur in a small cooled sample of the fluid. Allow the concentrated solution to stand for 12 hours, then filter off the crystals of the copper salt of glycine through a Buchner funnel, partially dry them by thorough suction with the water pump, and wash with dilute spirit (about 70 per cent.) followed by 90 per cent. alcohol.

Dissolve the copper salt in warm water, and precipitate the copper as sulphide with sulphuretted hydrogen, thus setting free the glycine. Filter off the copper sulphide, wash the precipitate thoroughly with hot water, and concentrate the filtrate until crystals of glycine begin to separate. If the copper sulphide tends to form a colloidal solution and is not completely retained by the filter-paper, this difficulty may be overcome by adding a little freshly precipitated, and well-washed aluminium hydrate to the solution prior to filtration.

Yield of glycine 50 to 60 grms.

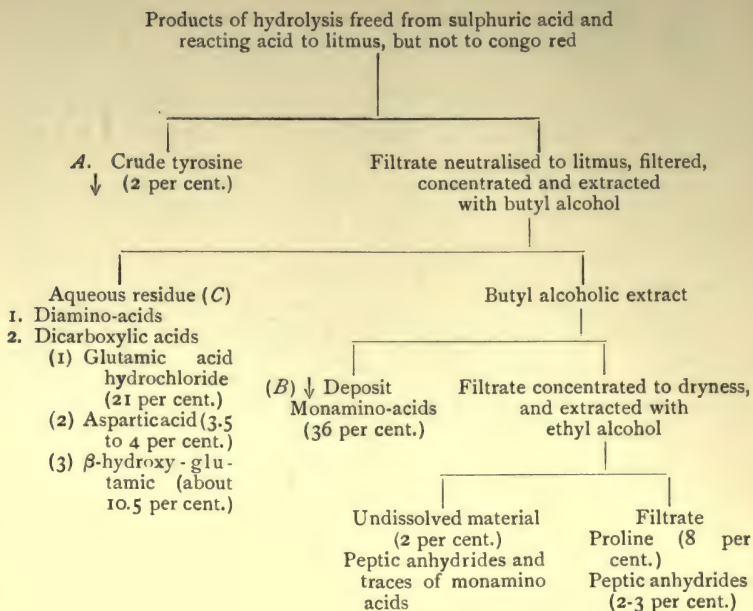
II. Dakin's method of extracting amino acids by means of a solvent (butyl alcohol) partially miscible with water.

100 grms. of pure caseinogen are heated with 500 to 600 grms. of dilute sulphuric acid (1 volume of the concentrated acid to 3 volumes of water) on the water bath in a flask provided with a reflux condenser until the greater part of the protein has dissolved. The hydrolysis is then continued by boiling the fluid in the flask on the sand bath for 12 to 16 hours. The solution is then diluted with water and the sulphuric acid quantitatively removed with barium hydroxide (about 600 to 700 grms.). The filtrate from the precipitate of barium sulphate, which reacts acid to litmus but not to congo red, is concentrated moderately, and about 2 per cent. of crude tyrosine allowed to crystallise out. The filtrate from tyrosine is made approximately neutral to litmus by the further addition of barium hydrate, again filtered, concentrated on the water bath until crude leucine begins to separate, and the whole mixture is transferred whilst still warm to the extraction apparatus (Kutscher-Steudel or other type), which will be figured in a later chapter. The amino acids from 100 grms. of caseinogen may be conveniently extracted in an apparatus holding about 350 c.c. It is preferable to arrange conditions so that the aqueous layer occupies three-quarters or five-sixths of the available space, and the volume of supernatant butyl alcohol is relatively small. The extraction flask should be of resistance glass, and the exposed surfaces of rubber connections should be reduced to a minimum. The extraction flask is heated over a sand bath preferably with rather high sides so as to reduce condensation in the flask, and the flame is adjusted so that a reasonably rapid flow of alcohol returns into the flask.

Cream coloured granular particles of amino acids soon separate out in the extraction flask, and the amount steadily increases. After one day's extraction, the extract is allowed to stand overnight, the separated amino acids are filtered off on a Buchner funnel, and washed with butyl alcohol and ether, the combined alcoholic filtrates being used for extraction on the second day. 36 hours are usually required for complete extraction, the time varying with the rate at which the butyl alcohol is boiled. The collected mixed amino acids are dried in vacuo over calcium chloride.

The following scheme summarises the outlines of the further procedure: ¹—

¹ The reader is referred to Dakin's paper, "The Biochemical Journal," vol. xii. pp. 290 to 317, for full details.



FURTHER TREATMENT OF THE PRODUCTS OF HYDROLYSIS.

Glutamic acid hydrochloride may be prepared from the aqueous residue (C). If desired, an aliquot portion of this residue may be reserved for the preparation of the diamino acids. The aqueous residue (C) is placed in a wide-mouthed bottle, cooled thoroughly, and saturated with dry hydrogen chloride. The latter is generated by placing about 200 c.c. of concentrated hydrochloric acid in a filtering flask closed with a rubber stopper, through which the stem of a separating funnel is passed. The side tube of the filtering flask is connected with a wash bottle containing concentrated sulphuric acid. A delivery tube from the wash bottle is immersed in the aqueous solution (C). Concentrated sulphuric acid is then dropped from the separating funnel into the flask containing the concentrated hydrochloric acid. The rapidity with which the hydrogen chloride is evolved is regulated by the rate at which the sulphuric is added. It is advisable to pass a fairly rapid current of hydrogen chloride into the solution. Crystals of glutamic acid hydrochloride soon separate out. When the fluid (C) has become saturated with the gas, the bottle is stoppered and left overnight in the ice chest. The crystalline mass is then mixed with an equal volume of ice-cold alcohol, and filtered off through asbestos, or through hardened filter-paper placed in a Buchner funnel, and drained as thoroughly as possible by suction with the water pump. The glutamic acid hydrochloride is purified by dissolving it in water, decolorising with animal

charcoal, filtering, and again precipitating by saturation with hydrogen chloride. The crystalline precipitate after being washed with ice-cold alcohol, and partially dried by suction, is finally transferred to a porcelain basin, and dried in vacuo over sticks of caustic soda.

After precipitation with phosphotungstic acid and recovery from this precipitate, the diamino acids may be separated from the other portion of (C) by the methods due to Kossel and his pupils. A description of these lies outside the scope of this book.

III. Preparation of cystine.

250 grms. of horse hair are mixed with 750 c.c. of concentrated hydrochloric acid in a flask (of $1\frac{1}{2}$ to 2 litres capacity) fitted with a reflux condenser. The mixture is heated on the water bath until the greater part of the hair has dissolved, and then for 6 hours on the sand bath. The greater part of the hydrochloric acid is then distilled off in vacuo, the round bottomed flask being heated on the water bath at 40° to 50° . The excess of hydrogen chloride can be most conveniently removed by distilling about 200 c.c. fractions of the fluid at each operation. More complete removal of the acid may be secured by dissolving the syrupy residue in water and repeating the distillation in vacuo. The residues are dissolved in about a litre of boiling water to which some animal charcoal is added, and the solution is filtered. The filtrate, which is still dark brown, is rendered strongly alkaline with ammonia, again heated with animal charcoal, and filtered. The alkaline filtrate is then concentrated in vacuo on the water bath. In a short time crystals, which mainly consist of tyrosine, separate out. These are filtered off on a small Buchner funnel, and the evaporation continued. A further separation of crystals, which consist of a mixture of cystine and tyrosine, occurs. These are also filtered off and collected separately from the first fraction. The filtrate is then acidified with acetic acid, and allowed to stand in the ice chest for 12 to 24 hours. The crystalline deposit, which separates, consists almost entirely of cystine. It is filtered off and purified by dissolving it in dilute ammonia and precipitating with acetic acid. Tyrosine separates out first when an ammoniacal solution of the two acids is concentrated, and a fairly complete separation may be secured by fractional crystallisation from ammonia.

SOME PROPERTIES OF CYSTINE.

(1) Cystine is very sparingly soluble in water (1 in 8840), alcohol, or ether, readily soluble in dilute mineral acids and in oxalic acid, and insoluble in acetic acid. It dissolves readily in alkalies, forming salts.

(2) Dissolve a little cystine in dilute ammonia, heat the solution, then acidify with dilute acetic acid, and allow the fluid to cool. Pipette off a drop of the sediment on to a microscopic slide, and examine the hexagonal plates of cystine with the high power of the microscope.

(3) Heat a small quantity of cystine with a solution of lead

hydrate in caustic soda. A black precipitate of lead sulphide separates out.

(4) Boil a little cystine with 10 per cent. caustic soda for a few minutes, cool, and add sodium nitroprusside. A reddish-violet colour indicates the presence of sodium sulphide.

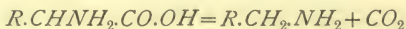
(5) Dissolve about 0.5 grms. (accurately weighed) of cystine in 50 c.c. of dilute ammonia and determine $[\alpha]_D = -142^\circ$. $[\alpha]_D$ of a 2 per cent. solution in dilute hydrochloric acid = -222° (approximate). The optical rotatory power of cystine is greater than that of any other known amino acid.

(6) Heat a solution of cystine in very dilute ammonia with Millon's reagent. A white precipitate is obtained, which does not become red on heating if the cystine be free from tyrosine.

IV. Some reactions of glycine and other amino acids.

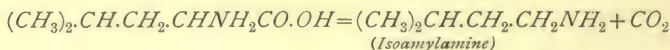
1. EFFECTS OF HEATING THE DRY SOLIDS.

(1) Heat a small quantity of glycine in a dry test-tube. It melts (m.p. 232° - 236°), simultaneously decomposing to yield a dark purple coloured fluid, which rapidly becomes brown and chars. The vapours given off have an alkaline reaction and ammoniacal odour (methylamine). The majority of the amino acids decompose on heating thus:—

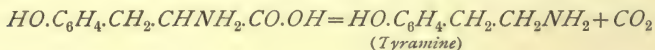


Other more complex and ill-defined changes occur concurrently. A better yield of methylamine is obtained from glycine, if an intimate mixture of barium hydroxide and the amino acid be heated.

(2) l-Leucine.—Heat gradually a small quantity of leucine in a dry test-tube. It yields a voluminous white sublimate consisting of the undecomposed acid, and alkaline vapours having the odour of isoamylamine.



(3) Tyrosine.—On heating, tyrosine melts (m.p. 295°) to form a clear fluid, which rapidly becomes yellow and then brown. Oily drops condense on the cooler sides of the test-tube. The odour of the vapour somewhat resembles that of phenol, but is more pungent. This experiment is better carried out in an evacuated and sealed tube. On standing, a large part of the oily deposit becomes crystalline. The crystals consist of p-hydroxy-phenylethylamine (tyramine)



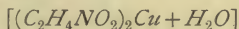
The foregoing reactions are all examples of decarboxylation of amino acids. Certain intestinal bacteria split off the carboxyl group from a number of amino acids. Thus tryptophane, arginine, lysine, and histidine are decarboxylated by certain bacteria yielding the bases—

indole-ethylamine, agmatine, cadaverine, and iminazole-ethylamine (histamine).¹

2. Dissolve a small quantity of glycine in a few drops of water (solubility 1 in 4). The solution is neutral to litmus and methyl red, and is sweet. Place a drop or two of the solution on a slide, concentrate slightly over the open flame, set aside to crystallise, and examine with the microscope. The crystals are well-defined prismatic needles belonging to the monoclinic system.

3. Heat a small quantity of glycine with absolute alcohol. It remains undissolved, but dissolves on the addition of a drop or two of concentrated hydrochloric acid. Glycine is insoluble in absolute alcohol, while its hydrochloride ($\text{CH}_2\text{NH}_3\text{Cl.CO.OH}$) is sparingly soluble. The solubility in alcohol of the homologous amino acids alanine, valine, and leucine increases with increasing molecular weight, while their solubility in water diminishes with increasing molecular weight. All are only very sparingly soluble in alcohol. Their hydrochlorides resemble that of glycine in being more soluble in alcohol than the free amino acids.

4. As an acid, glycine also forms salts with bases. Heat a little aqueous solution of glycine with excess of copper carbonate until effervescence ceases. Filter while hot, and place a drop of the dark blue filtrate on a slide. Blue needle-shaped crystals (solubility 1 in 174) of the copper salt



separate out on cooling. All the amino acids yield similar compounds, although some of their copper salts contain no water of crystallisation. The colour of the copper salts varies in depth with the percentage of copper. Thus the copper salt of leucine appears almost colourless on microscopic examination.

The amino acids also form silver salts. These are best prepared by adding slightly more than the equivalent amount of silver nitrate to a solution of the amino acid, and then drop by drop a saturated solution of barium hydrate as long as a white precipitate of the silver salt comes down. The addition of the barium hydrate is stopped as soon as the solution acquires a light yellow colour, indicating the commencing precipitation of silver oxide. A similar method is employed in separating the silver compounds of the diamino acids.

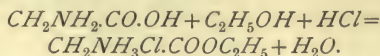
The sodium, potassium, and ammonium salts of the amino acids are all readily soluble in water. The fact that the calcium salts of the dicarboxylic acids are insoluble in alcohol has been utilised in the separation of these acids (Foreman).

5. Glycine also forms compounds with salts, e.g. with potassium chloride, barium chloride, and ferric chloride. Add a few drops of ferric chloride to a solution of glycine. A deep red colour is produced.

6. Glycine, as an acid, also forms esters with alcohols. Suspend 1 gm. of finely powdered glycine in 6 c.c. of absolute alcohol, saturate the solution with dry hydrogen chloride, fit the test-tube with a rubber

¹ For full details the reader is referred to "The Simpler Natural Bases," by G. Barger. Longmans, Green & Co.

stopper through a hole in which a glass tube is inserted to serve as a reflux condenser, and heat the fluid on the water bath for about 20 minutes. Remove the condenser, cool and stopper the test-tube, and leave in the ice chest. Crystals of the hydrochloride of the ethyl-ester of glycine separate out. This compound is distinguished from the corresponding derivatives of other amino acids by the readiness with which it crystallises from alcohol.



Filter off the crystals on a Buchner funnel, wash with a little ice-cold alcohol, and dry in vacuo over calcium chloride.

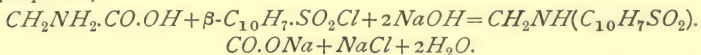
The free ester may be obtained from the hydrochloride by decomposing the latter with alkali and extracting the ester with ether, thorough cooling being essential to prevent saponification. For practical details of the method the reader is referred to the literature cited at the close of this chapter.

The esters of the amino acids can be distilled *in vacuo* practically, without decomposition, and E. Fischer has based on this fact a method for separating the amino acids by fractional distillation of their esters, the amino acids being recovered from the esters by hydrolysis. This method has extended our knowledge of the chemistry of the amino acids more than any other.

7. The amino group in glycine is highly resistant to the action of alkalies and acids. The amino acid may be boiled with 20 per cent. caustic soda without decomposition, while amides such as asparagine yield ammonia under the same conditions.

8. As amines, glycine and the other amino acids react with acid chlorides. Most of these compounds are very sparingly soluble in water, and have proved useful in isolating the amino acids. The compound which glycine yields with β -naphthalene sulphochloride may be prepared in the following way:—

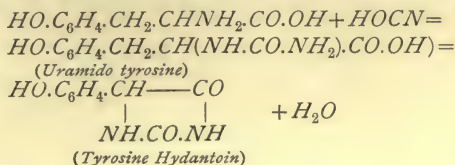
Dissolve 2 grms. of glycine in 26.7 c.c. of N. caustic soda (1 mol. of alkali to 1 mol. of glycine) placed in a bottle, add an ethereal solution of 12.1 grms. of β -naphthalene sulphochloride, stopper the bottle, and place it in a shaking machine. Add the same quantity of caustic soda thrice at intervals of about an hour. After 4 hours separate the aqueous solution from the ethereal one in a separating funnel, filter the former and acidify with hydrochloric acid. The oil (β -naphthalene sulphoglycine) thus precipitated soon becomes crystalline, and is then filtered off and purified by recrystallisation from boiling water. The compound consists of fine plates arranged in sheaves (m.p. 156).



9. The amino acids form uramido acids when their solutions are heated with urea or potassium cyanate. The uramido compounds are converted into hydantoin when boiled with hydrochloric acid.

PREPARATION OF TYROSINE HYDANTOIN.

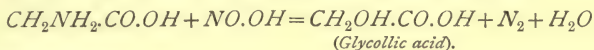
Heat 1 grm. of tyrosine suspended in 5 c.c. of water with about 0.5 grm. of potassium cyanate until a clear solution is obtained. Then boil with 10 c.c. of hydrochloric acid (1 part concentrated acid and 2 parts water) for a quarter of an hour. A colourless crystalline mass consisting of tyrosine hydantoin separates out (Dakin).



The preparation of the hydantoins has proved a valuable method of separating and identifying the amino acids (Dakin, *loc. cit.*).

10. Deamination of amino acids.

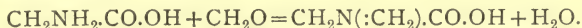
Acidify a 2 per cent. solution of sodium nitrite with acetic acid, and add to a dilute solution of glycine. Nitrogen is evolved, and the glycine is converted into the corresponding hydroxy acid.



This is a general reaction given by all amino acids having the amine group united to an aliphatic radicle. Van Slyke's method for the estimation of the amino acids is based on this fact.

Certain bacteria and fungi deaminate the amino acids. The products of bacterial action are generally ammonia and a fatty acid, less commonly a hydroxy acid. When, as a result of the action of bacteria or fungi, deamination is combined with decarboxylation, alcohols or hydrocarbons are obtained. Thus glycine may yield methane. Alanine, on deamination, yields lactic acid, and the latter, on decarboxylation, may yield ethyl alcohol. Leucine is deaminated and decarboxylated by yeast cells yielding isoamyl alcohol, one of the chief constituents of fusel oil (F. Ehrlich). Tyrosine is decomposed by intestinal bacteria yielding a number of aromatic acids, the final product being phenol. Histidine yields an unsaturated acid originally known as urocanic acid (Hunter).

11. The amino acids combine with formaldehyde to form methylene derivatives (Schiff).



The methylene derivatives are acid substances, and can be titrated with alkali. Sørensen has based a method for the estimation of amino acids on this fact.

The reader is referred to "The Chemical Constitution of the Proteins," by R. H. A. Plimmer, Longmans, Green & Co., and to Cole's "Practical Physiological Chemistry," pp. 67 to 99, for further details.

CHAPTER XI

THE LIVER AND PRODUCTS OF ITS ACTIVITY

I. GLYCOGEN—METHOD OF SEPARATION.

A RABBIT is fed with carrots, and, after ten hours, is killed by bleeding. The liver is excised as rapidly as possible. A small portion is set aside.¹ The remainder is chopped up, and thrown into five times its volume of boiling water, faintly acidified with acetic acid. The mixture is then filtered through calico, and the residue ground up with further quantities of water, and again extracted at 100° C. The extracts are united, and evaporated down to a small bulk, about 150 c.c. The concentrated extract is cooled, and freed from protein and gelatin by acidification with hydrochloric and the addition of Brücke's reagent (*i.e.* a saturated solution of mercuric iodide in 5 per cent. potassium iodide). The alternate addition of acid and Brücke's reagent is continued until a precipitate ceases to form. The solution is then filtered. Mix the filtrate with twice its volume of methylated spirit. The precipitate of glycogen which forms is allowed to settle, then filtered off and washed with diluted alcohol (2 vols. alcohol to 1 vol. water). Finally, it is washed with alcohol and ether, and dried. (*See* p. 31 for the tests for glycogen.) The portion of liver which was set aside may be extracted with hot water faintly acidified with acetic acid, and the filtrate tested for glucose and glycogen (*cf.* with extract of fresh liver).

¹ This portion should be kept for a short time at 37° to 40° C.

II. BILE.

INTRODUCTION.

The bile secreted by the liver cells becomes more concentrated during its passage along the bile ducts and during its stay in the bladder. The rise in concentration is due mainly to the absorption of water from it, but also in part to the addition of a glucoprotein (human bile) or phosphoprotein (ox bile) secreted by the cells lining the bile ducts and gall bladder. The bile collected from a fistula is even more dilute than that secreted under normal conditions, since many of the constituents of bile are normally absorbed from the intestine, and again excreted in the bile.

The following table (Hammarsten) gives the composition of human bladder and fistula bile:—

	Bladder Bile.	Fistula Bile.
Water	82.968	97.160
Solids	17.032	2.84
Mucin and pigments	4.191	0.910
Bile salts	9.697	0.814
Taurocholate	2.74	0.053
Glycocholate	6.957	0.761
Fatty acids	1.117	0.024
Fats	0.190	0.08
Cholesterol	0.9860	0.096
Lecithin	0.223	0.048
Soluble inorganic salts	0.288	0.8051
Insoluble inorganic salts	0.222	0.0411

The bile also contains traces of ethereal sulphates, urea, oxalic acid, and frequently salts of heavy metals, such as Zn, Cu, Mn. Traces of certain enzymes—diastatic, proteolytic and lipolytic, and katalase—are also present.

The cholesterol is kept in solution as an emulsoid colloid as a result of the solvent action of the bile salts. The pigment bilirubin and the products of its oxidation are faintly

acid substances, insoluble in water, but forming soluble salts with Na and K, and insoluble Ca and Ba salts. Part of the bilirubin is reduced to hydrobilirubin in the intestine. When oxidised with chromic acid it yields the same organic acids as hæmatin under similar conditions. Bilirubin is soluble in chloroform, in alcohol, and sparingly soluble in ether. According to Küster, it has the formula $[(C_{16}H_{18}N_2O_3)_x]$, x being probably = 2].

Either ox or sheep bile may be used for the following tests:—

1. PHYSICAL CHARACTERISTICS.—Note that the bile of the ox is a clear, viscid fluid of greenish-brown or green colour, having a bitter taste and peculiar aromatic odour.

2. REACTION is neutral or faintly alkaline.

Test with glazed litmus paper.

3. HEAT.

The fluid does not yield a coagulum on boiling.

4. MUCIN.

(a) *Acidify a small quantity of the bile with dilute acetic acid; a precipitate of a mucinoid nucleo-albumin separates out.*

(b) *To a small quantity of bile add methylated spirit; a bulky precipitate of the mucinoid nucleo-albumin separates out, along with some adhering bile pigment.*

5. GMELIN'S TEST FOR BILE PIGMENTS.

(a) *Place a little impure nitric acid¹ in a test-tube. Incline the test-tube, and pour bile slowly down the side of the tube, so that it forms a layer on the surface of the nitric acid.*

Note the following series of colours. The layer of bile in contact with the nitric acid has a yellow colour, due to the pigment *choletelin*. Above this band lies a red-coloured

¹ Nitric acid containing sufficient nitrogen peroxide for the test may be prepared by the addition of a small quantity of granulated zinc or a trace of sodium nitrite to the concentrated acid. As soon as all the zinc has dissolved, the acid is placed in a stoppered bottle.

layer, next to that a narrow band of violet, passing into blue (*bilicyanin*), still higher up in the test-tube, a green colour, due to *biliverdin*, and finally, nearest the surface, unchanged bile pigment, bilirubin. A precipitate is seen at the plane of junction of the acid with the bile, due to the separation of *bile acids* and *nucleo-albumin*.

(b) *The same may also be done in another way, namely, by mixing a little bile with impure nitric acid on a piece of white porcelain.*

The colours are then seen as a series of zones.

6. PETTENKOFER'S TEST FOR BILE SALTS.

(a) *Mix a little bile with two or three drops of a 10 per cent. solution of cane sugar.¹ Place in a test-tube some concentrated sulphuric acid (about half the volume of the bile used). Then incline the test-tube containing the sulphuric acid, and pour the bile, mixed with cane sugar solution, slowly down the side of the inclined test-tube, so that it forms a layer above the sulphuric acid.*

A precipitate of bile salts and mucin forms at the plane of junction of the fluids. Note the reddish-violet colour which develops in the bile lying immediately above the sulphuric acid.

Then mix the two fluids, preventing too great a rise of temperature by cooling the test-tube under the water tap. The fluid acquires a reddish-violet tint, which rapidly darkens to a purple colour on shaking up with the air.

(b) *Another method of doing the same test is to mix the bile, as before, with a little cane sugar. Then shake up the solution until a froth forms in the upper part of the test-tube. Pour a little concentrated sulphuric acid down the side of the tube. A purple colour develops in the froth where it has come into contact with the sulphuric acid.*

The test is due to the formation of a compound of furfural with the cholalic acid contained in the bile acids. The fur-

¹ Glucose may be used instead of cane sugar. It has the advantage of charring less readily than the latter.

fural is formed by the action of the sulphuric acid on the sugar. A drop of a saturated aqueous solution of furfural may be added to the bile instead of cane sugar, and the test carried out by either of the methods above described.

7. SURFACE TENSION TEST (MATTHEW HAY).

Place highly diluted bile in one test-tube, and water in another. Sprinkle a little flowers of sulphur on the surface of each fluid. The sulphur floats on the surface of the water, while it rapidly sinks in the solution of bile.

This test depends on the fact that bile salts reduce the surface tension of the water in which they are dissolved.

[I. PREPARATION OF GLYCOCHOLIC ACID.]

1. Add 10 c.c. of concentrated hydrochloric acid to 200 c.c. ox bile, placed in a stoppered vessel. Then add 25 c.c. of ether, and shake the mixture vigorously. Add a crystal of glycocholic acid, and allow the solution to stand in a cool place. A mass of crystals of glycocholic acid separates out. This is filtered off, and washed with cold water until the filtrate is colourless. The crystalline deposit is then transferred to a flask, and dissolved in as little boiling water as is necessary for its solution. The solution is then filtered hot. On cooling, glycocholic acid separates out in the form of fine needles.

[II. PREPARATION OF BILE SALTS (SODIUM GLYCOCHOLATE) — $C_{23}H_{36}(OH)_3.CO.NH.CH_2.CO.ON^a$ — AND TAUROCHOLATE — $C_{23}H_{36}(OH)_3.CO.NH.CH_2.CH_2.SO_2.ON^a$.]

Mix 500 c.c. bile with charcoal, and evaporate to dryness on the water bath. Transfer the residue to a flask, and extract with boiling methylated spirit for half an hour, using a reflux condenser. Cool, and filter the alcoholic solution. Distil off the methylated spirit, and extract the dry residue with the minimal amount of absolute alcohol necessary for its solution. Add to the alcoholic solution sufficient anhydrous ether to produce a permanent precipitate. On standing, the bile salts separate out as a crystalline precipitate.

N.B.—The ether used should be freed from water by

being left in contact with anhydrous calcium chloride for twenty-four hours, and then filtered.

1. Perform Pettenkofer's test with a solution of the bile salts. Dilute the purple fluid so obtained with glacial acetic acid, and examine it spectroscopically.¹

2. Examine the crystals of glycocholic acid microscopically.

3. Note the bitter taste of a solution of the salts.

4. To a small quantity of a solution of sodium glycocholate add a few drops of aqueous cupric sulphate. A precipitate of the cupric salt forms. Lead acetate also produces a precipitate of the lead salt.

III. CHOLESTEROL ($C_{27}H_{43}OH$).

Cholesterol may be prepared from gall-stones by extracting the finely powdered stones with ether. The ethereal solution is filtered, and evaporated to dryness. The cholesterol so obtained is sufficiently pure for the following reactions:—

1. It is quite insoluble in water, readily soluble in ether, chloroform, and hot alcohol.

2. Dissolve a little cholesterol in anhydrous ether. Examine microscopically the crystals which separate out on evaporation of the ether on a watch glass.

Note that they have the form of fine needles.

3. Dissolve some cholesterol in hot alcohol. Place a few drops on a watch glass, and examine microscopically the crystals which separate out in the form of fine rhombic plates, frequently having characteristic notched angles.

4. Place a cover-slip over some cholesterol crystals placed on a slide, and irrigate with a drop of sulphuric acid (5 vols. of concentrated sulphuric and 1 vol. water). Then place a drop of a solution of iodine in potassium iodide at the edge of the cover-slip. The crystals acquire

¹ Acid albumin gives a somewhat similar colour reaction, differing, however, in spectroscopic characters.

a reddish colour, owing to the action of the sulphuric acid. As the iodine reaches them, the red changes to a violet, and ultimately to a blue colour.

5. SALKOWSKI'S REACTION.

Dissolve some cholesterol in a little chloroform. Add an equal volume of concentrated sulphuric acid, and mix the two fluids by shaking. The chloroform rises to the top as a red-coloured layer, while the subjacent sulphuric acids shows a well-marked green fluorescence. Pour a little of the chloroform solution into a dry test-tube, and then add a drop of water. The chloroform solution is decolorised. On again adding concentrated sulphuric acid, the red colour reappears.

6. LIEBERMANN'S REACTION.

[Add ten drops of acetic anhydride to 2 c.c. of a solution of cholesterol in chloroform, placed in a dry test-tube. Then add a drop of concentrated sulphuric acid. The solution becomes red, then blue, and finally bluish-green in colour.]

GALL-STONES.

There are at least three main types—

(a) PIGMENT CHALK.

(b) CHOLESTEROL.

(c) CALCIUM CARBONATE AND PHOSPHATE.

But although they may be thus classified, it does not signify anything beyond the fact that the name applied refers to the main constituent. Thus, pigment chalk stones always contain more or less cholesterol, cholesterol ones more or less pigment and calcium salts, and the last-mentioned type always a small amount of pigment.

The first two types are those met with in man—

(a) Vary much in size, always dark-coloured (dark green to black), irregular in outline. They have a more or less

metallic lustre, and nearly always sink in water. They are common in the ox.

(b) Vary also much in size. They have, however, especially on section, a wax-like appearance, and when scraped give more or less the same impression. The nucleus is often a pigmented one, and around it the cholesterol is often laid down in concentric rings (often as alternate cholesterol and pigment chalk rings in large stones). They are often faceted, practically always much smoother than the first-mentioned ones, and, as a rule, float in water.

(c) In the third variety there is a minimum of pigment. These gall-stones are exceedingly rare in man. In the ox they are frequently met with as small, round, slightly pigmented granular clumps (gravel).

METHOD OF EXAMINATION.

1. *Place in a vessel of water, and see whether they float or sink.*

2. *With the finger nail scrape the surface. Note whether hard, granular, or wax-like. See if there be facetting.*

3. *Examine a section of one for appearance of concentric rings or radial striations.*

4.¹ *Powder half a dozen or so. Place in a flask, and extract as thoroughly as possible with about ten times the volume of ether. Shake for five to ten minutes. Test ethereal extract (a) and residue (b). Pour off ethereal extract (a), distil off ether, and test residue for cholesterol (p. 149). Drain off ether from residue (b), after washing it twice or thrice with ether; then shake up the residue with warm HCl (1 in 3). Test this acid extract (c) for calcium salts and copper, the former being detected in one portion by adding ammonium oxalate (after rendering the solution ammoniacal, and then adding acetic acid); the*

¹ Ethereal and acid extracts of gall-stones may be given out to the class for examination.

latter, in another portion, by addition of a few drops of potassium ferrocyanide solution (chocolate - coloured copper ferrocyanide).

[(*a*) Wash the residue from the above extract with water until Cl-free; then shake up thoroughly with a small quantity of CHCl_3 , the extraction being best carried out in a small flask (with condensing glass tube) on the warm water bath. The pigments are present in this extract.]

[(*a*) Take a drop or two of the extract on a slide, allow CHCl_3 to evaporate off, and examine residue microscopically.]

[(*β*) Take another portion, evaporate off CHCl_3 , touch residue with drop of impure $\text{HNO}_3(\text{HNO}_2)$. Note the play of colours (Gmelin's test).]

[(*γ*) Mix up in separating funnel the rest of the CHCl_3 , extract with 1 per cent. Na_2CO_3 ; the colour gradually disappears from the CHCl_3 , the pigment being taken up by the alkali. Run out the alkaline extract, pass stream of air through it; solution becomes green from biliverdin formation.]

CHAPTER XII

BLOOD

INTRODUCTION

BLOOD consists essentially of red and white corpuscles and platelets, suspended in a fluid—the plasma. On standing, it clots, owing to the separation of an insoluble protein—fibrin. The clot gradually contracts, expressing a fluid—the blood serum. If during the process of clotting the blood be whipped, the clot may be separated in the form of threads of fibrin, and one obtains a fluid—defibrinated blood—containing the corpuscles in suspension.

These changes may be summarised as follows:—

Circulating blood = plasma + formed elements (corpuscles and platelets).

Blood which has clotted, while at rest outside the vessels = clot (fibrin and formed elements) + serum.

Blood which has been whipped during clotting = fibrin + defibrinated blood (formed elements and serum).

The clotting or coagulation of shed blood may be delayed or prevented by cooling, collecting in paraffined vessels, by the action of neutral salts (*e.g.* magnesium sulphate), agents which remove the calcium ions [*e.g.* soluble oxalates, fluorides (1 vol. of 3 per cent. sodium fluoride to 9 vols. of blood), and citrates (1 vol. of 2 to 5 per cent. sodium citrate to 4 vols. of blood)], by hirudin (a substance extracted from the salivary glands of the leech) either after injection or on addition to shed blood, and by the injection of certain organic substances such as proteoses, certain snake venoms, and bacterial toxins.

Coagulation is accelerated by moderate heat up to about 40°, contact with foreign bodies, and by aqueous extracts of

various organs and tissues, especially of those rich in nucleoproteins.

The precise nature of the process is still not clearly understood, and can only be considered very briefly. Clotting consists in the conversion of a soluble protein known as fibrinogen into an insoluble protein,—fibrin, which on examination with the ultra microscope, is found to separate as fine acicular crystals. Many of these crystals appear to radiate outwards from clumps of agglutinated platelets, while others form in contact with the glass. The crystals later coalesce to form a meshwork of fine filaments, constituting a jelly enclosing the corpuscles, which contracts, squeezing out the serum. A substance known as thrombin or "fibrin ferment" has been found to be essential for the conversion of fibrinogen into fibrin. Thrombin appears to be a product of the disintegration of blood platelets, and is believed to be derived from an inactive precursor, prothrombin. An activating agent, thrombokinase, resulting from the disintegration of leucocytes, and other tissue cells, and calcium ions, appear to be essential factors in the conversion of prothrombin into thrombin.

GENERAL PHYSICAL PROPERTIES OF BLOOD.

The Specific Gravity varies considerably with age and sex. Considerable variations are also found in pathological conditions. The average value for the adult male is 1.058.

The Osmotic Pressure and Allied Properties.—The osmotic pressure is usually calculated from the depression (Δ) of the freezing point (which varies from -0.482° to -0.605°) by means of the formula :—Osmotic pressure in atmospheres=

$$\frac{\Delta \times 22.4}{1.86}.$$

The osmotic pressure of blood having the freezing point -0.56 is therefore 6.74 atmospheres, or about 5122 mm. of mercury. 0.9 per cent. solution of sodium chloride has an osmotic pressure practically identical with this value.

Viscosity.—The viscosity of blood at 38° referred to that of water as unity is 4.4 to 5.5. That of plasma or serum varies

from 1.78 to 2.09. The very high value for the blood as compared with the plasma is due to the presence of corpuscles, and is found to vary directly with their number. A 0.9 per cent. solution of sodium chloride has almost the same viscosity as water. The high value for serum or plasma is, therefore, due to the proteins. The viscosity is diminished by about 0.8 per cent. for each degree rise of temperature until the temperature for coagulation of the proteins is reached. The viscosity is increased by a rise in the CO_2 -content of the blood. The latter rise is to be regarded as a consequence of the swelling of the corpuscles owing to imbibition of water produced by carbon dioxide. In the determination of the viscosity of blood, coagulation is, usually, prevented by hirudin. With the onset of clotting, the rapid rise of viscosity which occurs soon renders further observation impossible.

The Electrical Conductivity of the Blood.—The alternating current is carried almost entirely by the ions of the inorganic electrolytes. “The corpuscles refuse passage to the ions, or permit them only to pass very slowly so that the corpuscles may, in comparison with the serum, be looked upon as non-conductors” (G. N. Stewart). The conductivity of the serum varies little; while that of defibrinated blood varies greatly, being increased when the blood is poor in corpuscles, and diminished when the blood is rich in corpuscles. The conductivity of the blood is consequently increased in anæmia. When the conductivity of the plasma or serum, and that of defibrinated blood are known, the volume of the corpuscles may be calculated by a formula given by G. N. Stewart.

General Chemical Characters of Blood.

The reaction of the blood will be considered in the quantitative section of this book.

Chemical Composition of Blood.—The most complete analyses of mammalian blood are given by Abderhalden, from whose paper the following figures are taken:—

	1000 grms. blood (pig) contain	1000 grms. of serum contain	1000 grms. of corpuscles contain
Water	790.565	917.610	625.61
Solids	209.435	82.390	374.38
Hæmoglobin . . .	142.2	...	326.82
Protein	46.61	67.741	19.19
Sugar	0.686	1.212	...
Cholesterol . . .	0.444	0.409	0.489
Lecithin	2.309	1.426	3.456
Fat	1.095	1.956	...
Fatty acids . . .	0.475	0.794	0.062
Phosphoric acid (from Nucleic acid) . .	0.0578	0.0218	0.1045
Na ₂ O	2.406	4.251	...
K ₂ O	2.309	0.270	4.957
Fe ₂ O ₃	0.696	...	1.599
CaO	0.068	0.122	...
MgO	0.0889	0.0413	0.150
Cl	2.690	3.627	1.475
Phosphoric acid (total)	1.007	0.1972	2.058
Inorganic acid . .	0.749	0.0524	1.653

A scrutiny of the table reveals the following facts:—

Sodium salts, calcium salts, and sugar only occur in the plasma and serum; while the corpuscles are relatively rich in potassium salts. In some animals, *e.g.* carnivora and ruminants, the corpuscles also contain sodium salts; while sodium is present only in the plasma of the blood of the horse and rabbit.

The relative quantities of plasma and corpuscles may be calculated from the percentage of any constituent present in the one and absent from the other. Thus the corpuscles contain about 0.16 per cent. of Fe₂O₃, the blood contains .0696 per cent. of Fe₂O₃, and the plasma (or serum) contains no iron. 100 grms. of blood, therefore, contain $\frac{.0696 \times 100}{.16}$ or 43.5 grms. of corpuscles, and 56.5 grms. of plasma or serum.

Proteins of the Plasma of Mammalian Blood.—The plasma contains from 5 to 8 per cent. of coagulable proteins consisting of fibrinogen (0.15 to 0.6 per cent.), serum globulin (3.8 per cent.), and serum albumin (2.5 per cent.). The amount of fibrinogen is subject to greater variation than that of either serum albumin, or globulin. The percentage of fibrinogen may rise to 1.6 or about eight times the normal amount in cases of leucocytosis or suppuration (A. P. Matthews).

Intermediate Products of Metabolism in the Blood.—The blood naturally contains varying amounts of the products of metabolism of the lipins and proteins.

The distribution of non-protein nitrogen in human blood under various conditions has been recently determined by Folin and Denis, with the results given in the following table (abbreviated):—

Milligrammes of Nitrogen per 100 grms. of Blood.

Conditions of Diet and Health.	Non-protein N.	Urea N.	NH ₃ N.	Uric Acid.	Creatinine and Creatine (mgms.)	Creatinine. (mgms.)
Normal, purine free, high N diet (Urinary N 24.9)	34	16	0.1	2.5	9.5	1.1
Uræmia	284	228	0.66	6.6	46.0	26

The blood also contains traces of the following enzymes:—(1) amylase, (2) sucrase or invertase, (3) glycolytic, (4) lipolytic, (5) proteoclastic enzymes (pepsin and trypsin, together with their anti-enzymes), and (6) catalase. Certain specific autolytic enzymes may also be formed in response to the injection of foreign proteins (Abderhalden and others).

The blood plasma is coloured faintly yellow owing to the presence of a small quantity of lutein. Bile pigments and other derivatives of blood pigment may also be found in it under pathological conditions.

The practical study of the phenomena of hæmagglutination and hæmolysis by foreign sera is better suited for a

course of histology or experimental physiology than for a chemical course, and is therefore omitted, although the factors involved are essentially chemical in their nature.

The chief protein constituents of the serum have already been studied. It is therefore only necessary to consider the blood plasma and defibrinated blood.

BLOOD PLASMA.

I. INFLUENCE OF SALTS UPON COAGULABILITY.

1. *Three volumes of freshly drawn blood are thoroughly mixed with 1 volume of a saturated solution of magnesium sulphate. By the admixture with magnesium sulphate the coagulation of the blood is prevented.*

2. *Freshly drawn blood is mixed with so much 3 per cent. potassium oxalate that the total fluid contains 0.3 per cent. of the salt. Note that the blood, containing a soluble oxalate, does not coagulate on standing.*

The plasma may be best separated from the corpuscles by centrifugalising. After centrifugalisation, the plasma is siphoned off from the red blood corpuscles.

II. EXPERIMENTS ON THE CONDITIONS INFLUENCING COAGULABILITY OF THE PLASMA BY FERMENTS. .

(a) *To a portion of the salted plasma (1) add nine times its volume of water and a few drops of a solution of fibrin ferment¹ (or thrombin) or blood serum. Place on the water bath at 30° C. A jelly-like clot of fibrin forms, which contracts on standing, expressing a clear fluid—the serum.*

(b) *To another portion of salted plasma, add one-*

¹ In order to prepare a solution of fibrin ferment, mix 1 volume of serum or defibrinated blood with 15 to 20 volumes of alcohol, and allow the mixture to stand for some weeks. Filter off the precipitate, dry it in the exsiccator, and extract the ferment from it with water (Schmidt).

tenth its volume of a 3 per cent. solution of potassium oxalate, and then dilute with nine volumes of water. Add a little blood serum, and place the fluid on the water bath at 30° C. No clotting occurs.

(c) To a portion of oxalate plasma add one-tenth its volume of a 2.8 per cent. solution of calcium chloride ($\text{CaCl}_2 + 6\text{H}_2\text{O}$), mix well, and place on the water bath at 30° C. Clotting occurs.

III. SEPARATION OF THE PROTEINS CONTAINED IN "OXALATE PLASMA" BY FRACTIONAL PRECIPITATION WITH AMMONIUM SULPHATE.

(a) Dilute 12 c.c. of oxalate plasma with 30 c.c. of water. Then add 20 c.c. of a saturated solution of ammonium sulphate. A precipitate of fibrinogen is produced. Filter off the precipitate with the aid of the aspirating pump.

(b) To 40 c.c. of the filtrate add 25 c.c. of a saturated solution of ammonium sulphate = a precipitate of serum globulin. Filtrate with the aid of the aspirating pump.

(c) Heat a small portion of the second filtrate to boiling point after rendering faintly acid with dilute acetic acid = a precipitate of serum albumin.

(d) Saturate another portion of the second filtrate with finely powdered ammonium sulphate = a precipitate of serum albumin.

The precipitates of fibrinogen and serum globulin may be dissolved in 1 to 2 per cent. sodium chloride, and the difference in their temperatures of coagulation observed. For this purpose, larger quantities of oxalate plasma are desirable. Fibrinogen coagulates at *circa* 55° C. Serum globulin coagulates at *circa* 70° to 75° C.

Fibrinogen may also be precipitated by half saturation with sodium chloride. Complete saturation with sodium chloride only partially precipitates serum globulin.

DEFIBRINATED BLOOD.

(a) REACTION.

Place a drop of blood on glazed red litmus paper, and wash the slip of paper with distilled water. A distinct blue spot is seen where the blood has been in contact with the paper.

(b) GUAIAIC TEST.

To half a test-tubeful of water add two or three drops of defibrinated blood, and mix thoroughly. Then add some tincture of guaiac, and a small quantity of an aqueous or, better, ethereal solution of hydrogen peroxide ("ozonic ether"). On mixing, the solution becomes blue, owing to oxidation of the guaiac resin.

The red blood corpuscles transfer the oxygen from the hydrogen peroxide to the guaiac resin.

(c) BENZIDINE TEST (Adler).

Mix one drop of blood with 50 c.c. of water. To a small portion of this diluted blood add a few drops of benzidine dissolved in glacial acetic acid, or in alcohol acidified with glacial acetic acid, and a few drops of hydrogen peroxide solution. The solution rapidly becomes blue. A control test may also be done with water instead of diluted blood. 1 part of blood in 100,000 parts of water can be detected by means of this test, which is therefore a very delicate one. Its delicacy is greatly lessened if the blood has been previously boiled. A number of substances also interfere with the test, rendering it less delicate. Thus 1 part of blood in 1000 parts of urine can scarcely be detected by this method. Ferric salts and dilute solutions of iodine also give a blue colour with the reagent.

(d) CATALYTIC ACTION.

To a small quantity of defibrinated blood¹ add twice its volume of a solution of hydrogen peroxide. An evolution of bubbles of oxygen occurs, due to the action of catalase.

¹ For this test, the blood may be diluted with at least nine times its volume of water.

(e) "LAKING OF THE BLOOD."

To a small quantity of defibrinated blood add a little ether and an equal volume of water, then mix thoroughly. By reflected light the mixture appears darker red than the original blood. When examined in a thin layer by transmitted light, it will be found to be transparent, owing to the fact that the blood pigment has been dissolved out from the red blood corpuscles.

(f) CRENATION OF THE RED BLOOD CORPUSCLES.

To a small quantity of blood in a test-tube, add one-fourth of its volume of a saturated solution of sodium chloride. The mixture becomes brighter red in colour and more opaque to transmitted light.

This is due to the outward passage of water from the red cells into the concentrated saline plasma, causing a shrinkage of the cells.

[(g) COAGULATION OF THE PROTEINS OF DEFIBRINATED BLOOD BY HEAT.]

Dilute 25 c.c. of defibrinated blood with 150 c.c. water in a beaker, and neutralise with dilute acetic acid. Bring to boiling point, stirring constantly. Acidify very faintly with dilute acetic acid. Filter from the brown-coloured coagulum. The filtrate should be colourless and transparent, but if too much acid has been used it will have a brownish tint, owing to the presence of acid hæmatin. Concentrate filtrate to about 20 c.c.

[(a) *Test a portion with Fehling's solution—reduction due to glucose.*]

If the blood has stood for some time, a considerable proportion of the glucose may have undergone oxidation, due to the presence of a glycolytic ferment. For this reason, it is best to use freshly obtained blood.

[(β) *Acidify another portion with HNO_3 and add a little AgNO_3 ; a white precipitate of AgCl separates out (soluble in NH_3).*]

PREPARATION OF CRYSTALS OF
OXYHÆMOGLOBIN.

1. FROM RAT'S BLOOD.

Mix a small quantity of rat's blood with an equal bulk of water and a few drops of ether. Shake thoroughly. Place a drop of the laked blood on a slide, cover, and examine with a microscope. Rhombic crystals of hæmoglobin gradually separate out.

[2. FROM SHEEP'S, DOG'S, HORSE'S, OR GUINEA-PIG'S BLOOD.]

To 20 c.c. of sheep's blood add 2 to 5 c.c. water and 2 c.c. ether. Mix thoroughly until the blood is completely laked. The process may be controlled by microscopic examination. To the laked blood add an equal volume of a saturated solution of ammonium sulphate, mix the fluids, filter immediately from the precipitate which forms. On standing overnight, large rhombic crystals will be found on microscopic examination of the sediment. Crystals of methæmoglobin may also be prepared by this method, if the laked blood be first shaken with a few drops of a solution of ferricyanide. If the solution of defibrinated sheep's blood in a half-saturated solution of ammonium sulphate be faintly acidified with acetic acid and allowed to stand overnight, large rhombic crystals of acid hæmoglobin separate out. Examine these microscopically and also with the microspectroscope. If the above methods be applied to guinea-pig's blood, characteristic tetrahedral crystals are obtained.

SPECTROSCOPIC EXAMINATION OF THE BLOOD
PIGMENTS AND THEIR DERIVATIVES.

PRELIMINARY PHYSICAL CONSIDERATIONS.

A small direct vision spectroscope may be used for the examination of the absorption bands of the blood pigments. In its simplest form this instrument consists of an outer brass

tube, having at one end a vertical slit, the width of which may be varied by a circular screw. Within this there slides a second tube, which is provided with a lens at the end next the slit, a combination of flint and crown glass prisms arranged alternately, and a circular opening at the other end for the eye of the observer. In its passage through the instrument, white light undergoes dispersion into its constituents without deviation of the light in the middle region of the spectrum. Direct the spectroscope towards the light from the window, adjust the width of the slit, and focus until the dark Fraunhofer's lines of the solar spectrum are distinctly seen. Then fix the spectroscope in a suitable holder, with the slit directed towards a luminous gas flame. The solutions of the pigments may be examined in test-tubes, in wedged-shaped glass bottles, or in vessels with parallel sides. When test-tubes are used, it must be remembered that they act as cylindrical lenses, and care must be taken that the light, after passage through the fluid in the test-tube, is focussed as a bright line on the slit. A description of the large Kirchhoff and Bunsen type of spectroscope, giving sufficient detail for practical work, would be beyond the scope of this book. Text-books of practical physics, *e.g.* W. Watson's "Practical Physics," pages 289-296, 305-315, Longmans, Green & Co., may be consulted. The position of absorption bands is usually stated in wave-lengths (λ) expressed in $\frac{1}{10^6}$ of a millimetre ($\mu\mu$).

Hilger's wave-length spectrometer is a very convenient instrument for spectroscopic work, on account of the ease and rapidity with which the position and limits of absorption bands can be determined. The telescope and collimator are fixed at right angles, and the prism table with compound prism is rotated by means of a micrometer screw fitted with a helical drum graduated in wave-lengths. The latter passes under an index, by means of which the wave-length of any line in an emission spectrum is read, as it comes into coincidence with the intersection of the cross wires of the eyepiece. The instrument is adjusted initially, so that the index gives the readings 589 and 589.6 for the two D lines, when these are successively

brought to coincidence with the cross wires. The reader is referred to Baly's "Spectroscopy" (pp. 57-59) for a description of the prism. Instructions are also supplied with the instrument.

Small direct vision spectroscopes with wave-length scale are also convenient instruments for less accurate work. For diffraction spectra the reader is referred to Watson's textbook (*loc. cit.*).

1. Oxyhæmoglobin (HbO_2).

On gradually diluting a solution of 1 volume defibrinated blood in 5 volumes of water, the following appearances will be seen on examination with a small direct vision spectroscope. Concentrated solutions of oxyhæmoglobin, which, to the naked eye, appear dark red, will be found to allow light to pass through the red region of the spectrum between Fraunhofer's lines C and D. On further dilution, light will also appear in the green region of the spectrum. Between these two regions a broad dark absorption band will be noted. On further dilution this band becomes resolved into two—a narrow apparently darker band α , immediately to the violet side of D, and a broader apparently fainter band β , lying to the red side of E. Introduce into the flame some sodium chloride, fused to a loop of platinum wire. A bright yellow line, the D or sodium line, will appear. Note the position of the absorption band α , in relation to D. An extension of the spectrum towards the violet will be also noted as a result of the dilution. Extremely dilute solutions of oxyhæmoglobin, which to the naked eye appear of a light yellowish-red tint, will still be found to show the two characteristic bands. The exact position of the bands may be localised if a spectroscope be used, provided with either a wave-length or an arbitrary scale.

Persistence of the absorption bands.—The bands can just be detected when blood diluted 1 in 5000 with water is examined in a layer 12 mm. thick.

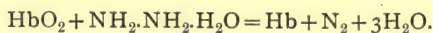
2. (Reduced) hæmoglobin (Hb).

(a) *To a solution of oxyhæmoglobin, diluted so as to show very distinctly the α and β bands, add a few drops of AMMONIUM SULPHIDE. In a short time the bright yellowish-red colour of the solution will become violet-red. On spectroscopic examination, a single dark absorption band, with ill-defined margins, will be found lying between D and E. The band may extend a little to the red side of D. The other margin of the band does not reach E. When ammonium sulphide is used as the reducing agent, a narrow absorption band will also be frequently seen in the red region of the spectrum. This band is due to a specific action of the ammonium sulphide, forming probably a sulphur compound of reduced hæmoglobin (sulphæmoglobin).¹*

Shake up the solution of reduced hæmoglobin with air and examine with the spectroscope. The two absorption bands of HbO_2 will again be seen. On standing, the HbO_2 is again reduced to Hb.

The additional band in the red is not seen if STOKES' REAGENT is used as the reducing agent. The latter reagent has also the advantage of acting more quickly than ammonium sulphide. For its preparation, make up a 2 per cent. solution of ferrous sulphate. To this add 2 to 3 per cent. of tartaric acid. This solution can be kept for a considerable time. Before use, a small quantity placed in a test-tube is rendered just alkaline with ammonia. Add a few drops of the alkaline solution to a solution of oxyhæmoglobin. Note the rapid change in colour and the appearance of the single band of reduced hæmoglobin on spectroscopic examination.

[(b)] HbO_2 may also be reduced to Hb by hydrazine hydrate. The reaction which occurs may be represented by the equation—



The volume of nitrogen set free from the hydrazine

¹ Hurltley and Clarke, "Journal of Physiol.," xxxvi. 62, 1907.

hydrate is equivalent to the amount of oxygen originally combined as HbO_2 , and upon this fact a method has been based for determining the oxygen capacity of hæmoglobin (Buckmaster).

Dilute one drop of blood with 5 c.c. of water, add one drop of hydrazine hydrate (50 per cent.), and examine the solution of (reduced) hæmoglobin with the spectroscope.

(c) Sodium hydrosulphite (10 per cent.) solution may also be used to convert oxyhæmoglobin into reduced hæmoglobin.

Dilute one drop of blood with 5 c.c. of water, add one drop of sodium hydrosulphite, and examine with the spectroscope.

[(d)] Oxyhæmoglobin dissociates *in vacuo* into (reduced) hæmoglobin and oxygen.

Dilute two drops of blood with 10 c.c. of water in a test-tube (A). Transfer half this solution to another test-tube (B). Fit (A) with a rubber stopper through a hole in which a glass tube provided with a stopcock is inserted. The stopcock being open, the test-tube (A) is heated in the water bath to $40^\circ - 45^\circ$. The glass tube is then connected with the water-pump, and the test-tube evacuated. A good deal of frothing occurs. This may be controlled by partially closing the stopcock. When evacuation is complete, close the stopcock, detach it and the test-tube from the pump, and examine the contents with the spectroscope, comparing with the solution in (B). Solution (A) gives the single band of Hb, while (B) naturally still shows the two bands of HbO_2 .

[(e)] *Persistence of absorption bands on dilution.*—The single band of Hb is just perceptible in a layer 10 mm. thick when the pigment has been prepared from a 1 in 500 dilution of blood with water.

3. Carbonic oxide hæmoglobin.

This pigment may be prepared by saturating defibrinated blood with carbonic oxide. The gas may be readily

obtained by the action of concentrated sulphuric acid upon formic acid.¹ The stream of gas should be allowed to pass through a wash bottle containing caustic soda before reaching the defibrinated blood.

(a) The solution containing carbonic oxide hæmoglobin has a brighter, more carmine-red tint than that of oxyhæmoglobin. A dilute solution of oxyhæmoglobin appears yellowish-red, while a solution of carbonic oxide hæmoglobin of the same strength has a distinct bluish-red tint. This difference is due to the fact that solutions of carbonic oxide hæmoglobin absorb less of the light in the blue and violet region of the spectrum than solutions of oxyhæmoglobin of corresponding strength. A dilute solution of carbonic oxide hæmoglobin shows two bands, closely resembling in position and character the corresponding bands of oxyhæmoglobin, but situated a little nearer the violet end of the spectrum. Note that the *a* band is farther separated from the *D* line than the *a* band of oxyhæmoglobin; and also note whether any differences can be made out as to the respective breadths and degrees of absorption in the absorption bands of the two pigments.

(b) To some dilute solution of carbonic oxide hæmoglobin add a few drops of ammonium sulphide, hydrazine hydrate, sodium hydrosulphite, or of Stokes' solution. Note that the two bands remain unaltered in position and character.

(c) To some blood saturated with carbonic oxide add half its volume of concentrated caustic soda solution. The blood saturated with carbonic oxide yields a bright red precipitate, while normal blood, when similarly treated, gives a dark brown coloration. After standing, the difference becomes less marked.

[(d)] Some additional reactions of carbonic oxide hæmoglobin.

Add 4 drops of defibrinated blood to 10 c.c. of

¹ A current of ordinary coal-gas may be passed through the blood instead of pure carbonic oxide.

water, mix thoroughly and divide the solution into two equal portions (A) and (B). Saturate (A) with coal-gas. Add to (A) and (B) dilute acetic acid (about 5 drops of 10 per cent. acetic) and potassium ferrocyanide solution (about 1 to 2 drops). (A) yields a carmine-red precipitate, while (B) gives a brown precipitate.

This reaction will be better understood when the derivatives of hæmatin have been studied. HbO_2 is decomposed by the acid and potassium ferrocyanide to yield globin (a colourless protein) and (oxy)hæmatin (a brown pigment). Globin and the proteins of the serum are precipitated, carry down the pigment with them as a loose adsorption compound, and are therefore coloured brown. HbCO is decomposed in a similar way, yielding globin and carbonic oxide-hæmatin (or CO-hæmochromogen), which is a carmine-red coloured pigment. $\text{HbCO} = \text{Globin} + \text{HtCO}$ (which colours the protein ^(Carmine-red) precipitate bright red). Since HtCO is decomposed by oxygen yielding (oxy)hæmatin and free CO, the carmine-red precipitate becomes brown on exposure to the air. Test (c) may be explained in a similar way.

A number of other protein precipitants, e.g. tannin, trichloroacetic acid, salicyl sulphonic acid and others may be used instead of acetic acid and potassium ferrocyanide.

[(e)] HbCO dissociates *in vacuo* into Hb and CO, but the compound of Hb with CO is much more stable than that with O_2 . If experiment 2.d. be repeated with a solution of HbCO great difficulty will be found in obtaining the spectrum of Hb, partly owing to the incomplete vacuum obtainable with the water pump. Hb also forms a compound with NO, which is even more stable than that with CO.

(f) The intensity of the absorption bands of HbCO is about the same as that of HbO_2 .

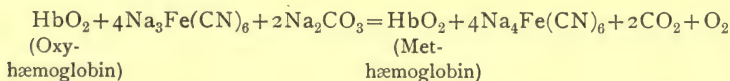
4. Methæmoglobin.

(a) Dilute 2 to 3 drops of blood with about 5 c.c. of water, and add a few drops of a concentrated solution of

potassium or sodium ferricyanide (these should be freshly prepared). Shake the mixture vigorously. Note that the solution becomes reddish-brown in colour, the particular shade depending upon the reaction. Dilute a little of this solution in a test-tube until a dark absorption band is seen, lying to the red side of D. On further dilution, two faint bands will be seen lying between D and E. The two latter bands closely correspond in position to the α and β bands of oxyhæmoglobin. A fourth band may also be seen lying in the bluish-green between b and F. The last band can be much better seen if the solution of the pigment be examined with the aid of a large Kirchhoff and Bunsen spectroscope.

(b) *To a small quantity of the dilute solution of methæmoglobin add a few drops of ammonium sulphide. The absorption bands of methæmoglobin will be replaced at first by the two bands of oxyhæmoglobin (or the two bands of alkaline methæmoglobin, the third fainter band of that pigment being also sometimes seen), and ultimately by the single band of reduced hæmoglobin.*

During the conversion of oxyhæmoglobin into methæmoglobin there is given off a volume of oxygen equal to that originally combined with the hæmoglobin present. The course of the reaction is probably to be represented by the following equation :—



The CO_2 may be absorbed by means of ammonia added to the blood (Haldane, see p. 339).

The quantity of oxygen yielded by one gramme molecule (mol) of oxyhæmoglobin (*i.e.* the quantity of HbO_2 containing 55.85 grms. or one gramme atom of iron) on conversion into methæmoglobin has been found to be 22,394 c.c. The ratio, $\frac{22,394}{55.85} = 401$ c.c., is known as "the specific oxygen capacity." One is therefore justified in employing the

symbols Hb and HbO_2 to represent the molecules of (reduced) hæmoglobin and oxyhæmoglobin.

Other reagents may be used in the preparation of methæmoglobin. The chief of these are nitrites, potassium permanganate, potassium chlorate, and hydrogen peroxide; thus, for example—

[(c) *To a 1 in 5 solution of defibrinated blood add two drops of amyl nitrite. Shake the mixture thoroughly, dilute, and examine with the spectroscope.*]

The previous description applies to the spectrum of methæmoglobin in neutral solution.

(d) *If a solution of methæmoglobin be rendered faintly alkaline by the addition of a few drops of ammonia, alkaline methæmoglobin is formed. The solution acquires a redder tint, and shows, on spectroscopic examination, two bands between D and E, somewhat resembling the absorption bands of oxyhæmoglobin. The β band is, however, darker than the α band. A third, fainter, band lies immediately to the red side of D.*

(e) Carbonic oxide hæmoglobin may also be converted into methæmoglobin.

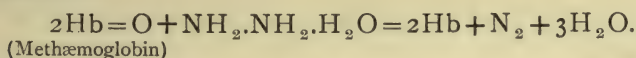
Dilute 2 drops of blood with 5 c.c. of water, pass a stream of coal-gas through the solution, then add a few drops of a saturated solution of potassium ferricyanide, and mix thoroughly. The conversion of HbCO into methæmoglobin takes place more slowly than that of oxyhæmoglobin, but the solution will ultimately be found to show the absorption spectrum of methæmoglobin.

[(f)] Methæmoglobin does not dissociate *in vacuo* into (reduced) hæmoglobin and oxygen. Carry out experiment 2 (d). The result is negative.

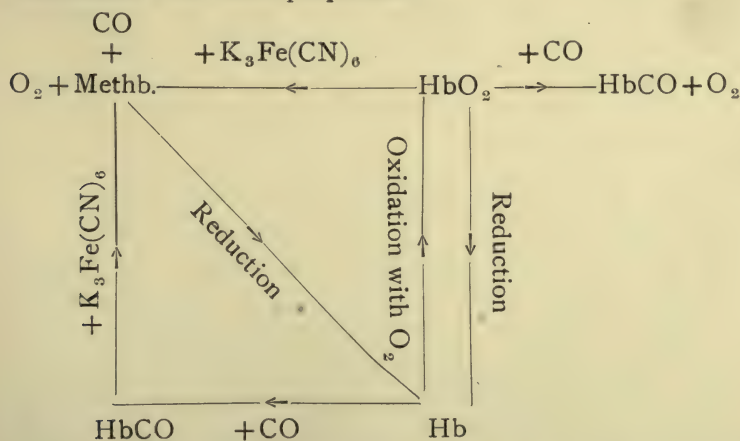
[(g)] *Intensity or persistence of absorption bands. Neutral methæmoglobin prepared from blood diluted 1 in 300 shows a very faint band in the red, just perceptible in a 10 mm. layer. Alkaline methæmoglobin shows 2 absorption bands when examined in a layer 11 mm. thick (dilution 1 in 1000 of blood).*

In order to explain the differences in the optical and chemical characters of the two compounds of oxygen with hæmoglobin, known as oxyhæmoglobin, and methæmoglobin, Haldane has suggested that oxyhæmoglobin should be represented by the formula $\text{Hb} \begin{smallmatrix} \diagup \text{O} \\ \diagdown \text{O} \end{smallmatrix}$, while the more stable oxygen compound, methæmoglobin, may be represented by $\text{Hb} \begin{smallmatrix} \text{O} \\ \parallel \\ \text{O} \end{smallmatrix}$.

More recently, Buckmaster has shown that, when methæmoglobin is converted into Hb by the action of hydrazine hydrate, the amount of nitrogen obtained is only half that liberated by the action of hydrazine hydrate on an equivalent amount of HbO_2 . This result would indicate that methæmoglobin should be represented by the formula $(\text{Hb}=\text{O})$, and the reaction with hydrazine hydrate by the equation—



The pigments described in 1 to 4 are all closely related to one another and are interconvertible. The following scheme summarises their chief properties :¹—



¹ This method of representation was suggested by the more comprehensive scheme given by Cole ("Text-Book," p. 242).

5. Action of acids on oxyhæmoglobin.

[(a) *Render diluted defibrinated blood (1 in 30) faintly acid by the addition of a little .4 per cent. hydrochloric acid. The solution soon acquires a brown colour, and, on spectroscopic examination, a band is seen in the red, closely resembling the corresponding band of methæmoglobin, but lying a little nearer the red end of the spectrum. Neutralise the solution with a few drops of dilute ammonia, and shake thoroughly. On spectroscopic examination, the bands of alkaline methæmoglobin will be seen. On the addition of a few drops of Stokes' solution, the bands of methæmoglobin will be replaced by the single band of reduced hæmoglobin (J. A. Menzies and others).*]

The pigment formed by the action of very dilute acids on oxyhæmoglobin has been termed *acid hæmoglobin*. By more prolonged action of acids, the oxyhæmoglobin is decomposed into the pigment (*oxy*)*hæmatin* and the protein globin. 100 grms. of (*oxy*)hæmoglobin yield 94.09 grms. of globin, 4.47 grms. of (*oxy*)hæmatin, and 1.44 grms. of other substances, including fatty acids (Lawrow). According to Willstaetter, (*oxy*)hæmatin has the empirical formula $(C_{33}H_{33}N_4O_5Fe)$. Oxyhæmoglobin is dextro-rotatory ($[\alpha]_c = +10.4^\circ$ —Gamgee and Hill); while the protein constituent globin is lævo-rotatory ($[\alpha]_c = -54.2^\circ$).

(b) *ACID (OXY)HÆMATIN.*—To some diluted defibrinated blood (1 in 5) add one-fourth its volume of 33 per cent. acetic acid. Mix the solutions thoroughly, and heat on the water bath at 40° to 50° (boiling produces marked turbidity) for at least five minutes. Dilute a small quantity of the solution and examine spectroscopically. A distinct absorption band will be seen in the red lying between C and D. The pigment formed is acid (*oxy*)hæmatin. Render the remainder of the solution faintly alkaline by the gradual addition of dilute caustic

soda, and then filter. To the filtrate add some ammonium sulphide or Stokes' solution.¹

On spectroscopic examination, the solution will be found to show the two characteristic bands of *hæmochromogen* or (reduced) *hæmatin*. The aqueous solution of acid *hæmatin* is, as a rule, more or less turbid. In acid alcohol or ether, *hæmatin* dissolves to form a clear solution, better fitted for spectroscopic examination than the aqueous one.

(c) The foregoing method is employed when one desires to prove that acid oxy*hæmatin* can be converted into an alkaline solution of (reduced) *hæmatin*.

A solution of acid (oxy)hæmatin of suitable concentration for examination with the spectroscope may be more simply obtained by mixing 4 or 5 drops of blood with 5 c.c. of 30 per cent. acetic, or preferably of glacial acetic acid, and heating on the water bath until the pigment has dissolved.

(d) *To some defibrinated blood add circa half its volume of glacial acetic acid, and at least twice its volume of ether. Mix the fluids thoroughly. The acid ethereal solution of hæmatin rises to the top. Part may be poured off, filtered and examined spectroscopically. It may be diluted, when necessary, with acid ether (1 part of glacial acetic acid to 2 parts ether).*

The solution shows a well-marked band in the red between C and D, lying nearer C than the corresponding band of methæmoglobin. Between D and F lies a broad band with ill-defined margins.

On dilution, the latter band becomes resolved into two bands—one, the narrower and fainter, lying in the light green immediately to the red side of E; the other, broader and darker, lying in the green between b and F. A fourth,

¹ The following method is a better one than that described. Add about 10 drops of a saturated aqueous solution of oxalic acid to half a test-tubeful of diluted blood (1 in 5), mix the solutions, place the tube in a water bath at 50°, and gradually raise the temperature of the water to 100° C. Dilute a small quantity of the solution, examine spectroscopically, then render alkaline with NH_3 and reduce with ammonium sulphide.

very faint band, may also be made out, lying on the violet side of D.

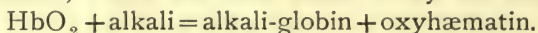
(e) A solution of (oxy)hæmatin in acid alcohol may be obtained by the following method :—

[To some defibrinated blood add sufficient alcohol to precipitate the proteins. Filter off the precipitate and extract it with alcohol containing oxalic, or 1 per cent. sulphuric acid. A dark brown solution of hæmatin in acid alcohol will thus be obtained.]

(f) *Intensity of absorption spectrum.*—The absorption band in the red is just detectable when solutions of acid (oxy)hæmatin, corresponding in concentration to a dilution of 1 in 200 of blood, are examined in a layer 11 mm. thick.

6. Alkali-hæmatin (or oxyhæmatin).

Oxyhæmoglobin is decomposed on heating with alkali into its protein component, globin, and a pigment (oxy)hæmatin, which are both dissolved by the alkali



(a metaprotein).

The alkali, naturally, also converts the proteins of the serum into alkali metaproteins.

To a small quantity of diluted defibrinated blood (1 in 5) add half its volume of concentrated caustic soda; mix and heat gradually, nearly to the boiling point. Cool the solution and shake up with the air, prior to examination with the spectroscope.

Alcoholic solutions of the pigment give a more distinct absorption spectrum. Add 10 drops of 10 per cent. caustic soda to 5 c.c. of methylated spirit, then add 5 to 6 drops of blood, heat on the water bath, filter, and examine the filtrate with the spectroscope, after shaking up with the air to ensure full oxidation of the pigment.

The latter precaution is necessary, since, by the action of the alkali on defibrinated blood, reducing substances are formed which convert part of the alkali hæmatin into hæmochromogen. On spectroscopic examination the greenish-

brown solution shows a broad faint absorption band lying to the red side of D. The band extends also a short distance towards the violet side of D.

7. (Reduced) alkali hæmatin or hæmochromogen in alkaline solution.

(1) *Dilute the solution of alkali-(oxy)hæmatin until the absorption band is no longer visible; then add a few drops of Stokes' solution, ammonium sulphide, hydrazine hydrate, or sodium hydrosulphite.*

The greenish-brown tint of the solution of alkali-(oxy)-hæmatin changes to the bright red colour of a solution of hæmochromogen. On spectroscopic examination a very dark, sharply defined, narrow band will be found lying almost midway between D and E. A much fainter band will also be found lying in the green between E and *b*. On dilution, the latter band disappears first.

[(2) Carboxyhæmatin (or carboxyhæmochromogen.)]
(Hoppe-Seyler and others).

Add 6 to 8 drops of blood to 20 c.c. of water, then add 10 to 15 drops of 10 per cent. caustic soda, gradually heat to the boiling point, then filter. Divide the filtrate into two equal portions (A) and (B), and add to each about half its volume of ammonium sulphide or a few drops of 10 per cent. sodium hydrosulphite solution. Fit test-tube B with a rubber stopper, remove the stopper, pass a stream of coal-gas, or, better, of pure carbon monoxide through the test-tube, maintaining the current of gas during the withdrawal of the delivery tube, and close the test-tube with the stopper as rapidly as possible. Shake up the contents of (B), allow the test-tube to stand for a minute or two, then examine the contents with the spectroscope and compare with the absorption spectrum of (A). (B) shows two absorption bands of nearly equal intensity, the band (β) lying nearer the violet end of the spectrum being slightly fainter than the band (α), which lies immediately to the violet side of the D line. The absorption

spectrum of (B) therefore closely resembles that of carbonic oxide hæmoglobin, and is due to a compound of (reduced) hæmatin with carbon monoxide.

This compound contains one molecule of carbon monoxide for each atom of iron (Pregl), and has, therefore, the same specific carbon monoxide capacity as HbCO . It differs from HbCO in being unstable in the presence of free oxygen, becoming rapidly converted into (oxy)hæmatin on exposure to the air. The carbon monoxide compound has a bright red colour, while (oxy)hæmatin is brown.

The amount of oxygen, which is removed in the reduction of (oxy)hæmatin to (reduced) hæmatin is uncertain. Since hydrazine hydrate reduces (oxy)hæmatin in alkaline solution, and the amount of nitrogen set free is equivalent to the oxygen removed, the method employed by Buckmaster might be utilised in determining the specific oxygen capacity of hæmatin.

Ham and Balean have found that "in the conversion of HbO_2 into acid hæmatin by the action of acids, only half the amount of replaceable oxygen as obtained by the ferricyanide method is liberated." This result indicates that (oxy)hæmatin probably only contains half as much oxygen as the equivalent amount of HbO_2 .

In acid solution reduced hæmatin is very unstable, the iron being readily detached even by weak acids and hæmatoporphyrin formed.

The appearance of the spectrum of hæmochromogen, produced by the action of reducing agents upon alkali hæmatin, is dependent upon the presence of certain impurities, these including ammonia and its salts, protein or other amine compounds. The following experiments will demonstrate this:—

[(a) Place small quantities of pure hæmatin, dissolved in dilute NaOH , in two test-tubes. To the one portion add a few drops of 10 per cent. glucose solution; to the other an equal volume of a solution of alkali albumin, and then a few drops of the glucose solution. Heat both solutions.]

Note that the latter acquires a bright red colour, while

the former¹ scarcely alters its tint. Examine both solutions spectroscopically. That containing alkali albumin will be found to show the spectrum of hæmochromogen, while the solution containing glucose alone shows at most the faint band of alkali hæmatin. In both cases reducing substances are formed by the action of the alkali upon glucose, but that these are not sufficient in themselves to produce hæmochromogen, is shown by the fact that, until such a body as alkali albumin is added, the spectrum remains that of alkaline hæmatin.

[(b) Add a trace of aluminium powder to a solution of pure hæmatin in dilute NaOH. Note that there is no apparent alteration in colour. Heat cautiously. Wait until the evolution of hydrogen slackens. Then examine spectroscopically.]

The bands of hæmochromogen are not present. The colour of the solution also remains unaltered.

[(c) Add a trace of aluminium powder to a solution of pure hæmatin in dilute ammonia, heat gradually to the boiling point, then cool.]

The solution acquires a bright red colour, and shows the spectrum of hæmochromogen.

8. Hæmatoporphyrin.

Pure crystalline hæmatoporphyrin prepared by the action on hæmin of a solution of hydrobromic acid in glacial acetic acid (sp. gr., 1.41) has the empirical formula ($C_{33}H_{38}N_4O_6$), according to Willstätter. The forms of hæmatoporphyrin or porphyrins prepared by other methods probably vary in composition although closely resembling one another in their optical and many of their chemical properties.

To 10 c.c. of concentrated sulphuric acid, placed in a beaker, add a few drops of defibrinated blood, stirring the mixture thoroughly after the addition of each drop. A purple solution of hæmatoporphyrin or iron free hæmatin,

¹ The slight alteration in tint, which also takes place in the former solution, is due to the formation of coloured products by the action of the alkali upon the glucose (*cf.* Moore's test).

showing a red fluorescence, is obtained. Take a small quantity of this solution in a test-tube. Dilute it with glacial acetic acid, and examine with the spectroscope.

The foregoing method is adopted, when an alkaline solution of hæmatoporphyrin is to be prepared afterwards.

A solution of hæmatoporphyrin of suitable concentration for direct spectroscopic examination may be more simply prepared by adding one drop of blood to about 5 c.c. of sulphuric acid placed in a dry test-tube, and mixing the contents thoroughly by transferring them to a second dry test-tube.

(a) ACID HÆMATOPORPHYRIN.

In presence of free mineral acid, a solution of hæmatoporphyrin shows two well-marked absorption bands—a narrow dark absorption band lying in the orange between C and D, close to D, and a second broader and darker band lying in the yellow-green, nearly midway between D and E. The margin of the latter band lying towards the violet end of the spectrum is sharp and well-defined; while the margin towards the red end of the spectrum is connected with an area of fainter absorption, which ends in a darker margin a little to the violet side of D.

(b) ALKALINE HÆMATOPORPHYRIN,

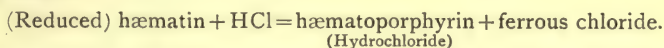
Pour the greater part of the acid solution of hæmatoporphyrin into excess of distilled water. Cool the solution and then gradually add caustic soda, stopping short while the solution is still acid. A pigmented precipitate separates out, containing the greater part of the hæmatoporphyrin. Precipitation may be rendered more complete by the addition of sodium acetate. Filter off the precipitate and dissolve it in a small quantity of dilute caustic soda.

The brownish-red solution thus obtained shows four absorption bands—a narrow band (α) in the red, about midway between C and D; a second (β) broader and darker band in the green, lying a little to the violet side of D; a third

band (γ) also situated in the green, between D and E, and extending a little to the violet side of E. A fourth broad and dark band (δ) lies at the junction between the green and blue, extending from b to F. Of these bands (a) is much the faintest, and disappears first on dilution.

[9. Some products of the reduction of (oxy)hæmatin in acid solution.]

We have already seen that when HbO_2 is decomposed by the action of acids, it yields globin and acid (oxy)hæmatin. When Hb is decomposed in a similar way, globin and hæmatoporphyrin are obtained (Hoppe-Seyler and Laidlaw), the iron being detached from the hæmatin molecule. Probably (reduced) hæmatin in acid solution forms an intermediate stage; but this substance is very unstable, and is readily decomposed by dilute mineral acids into hæmatoporphyrin and an iron salt, thus—



The preparation of solutions of (reduced) acid hæmatin is somewhat too difficult for class work and is therefore omitted.

(1) Add 0.5 gm. of stannous chloride, 7 drops of concentrated hydrochloric acid, and a few granules of tin (preferably precipitated tin) to about 10 c.c. of methylated spirit. Then add gradually 10 to 15 drops of blood, mix thoroughly, heat nearly to the boiling point in the water bath for 15 minutes, then filter. Dilute a few drops of the filtrate with alcohol and examine with the spectroscope. The solution is bright red and shows the two bands of acid hæmatoporphyrin.

Dissolve 2 grms. of anhydrous potassium carbonate in 2 c.c. of water, gradually add this solution to the filtrate, heating the mixture on the water bath after each addition of alkali until the evolution of CO_2 slackens, and the supernatant alcoholic solution has become red-brown and reacts alkaline to litmus paper. Add a few granules of anhydrous potassium carbonate to the mixture, heat, cool, and pipette or decant the supernatant alcoholic solution into a dry test-tube. Shake up the decanted alcoholic solution with a few granules of anhydrous potassium carbonate and again filter. Dilute a small quantity of the filtrate with alcohol and examine with the spectroscope. The 4-banded absorption spectrum of hæmatoporphyrin in alkaline solution will be readily identified.

(2) Preparation and properties of the zinc and copper compounds of hæmatoporphyrin.

Dilute a small quantity of the filtrate with alcohol, add a little concentrated ammonia, and a trace of a zinc salt, and heat the solution on the water bath for a few minutes (3-5). The colour of the solution changes from reddish-brown to bright red and shows the 2-banded absorption spectrum of so-called metallic hæmatoporphyrin,

described by Garrod and others. The two absorption bands closely resemble those of oxyhæmoglobin in position and character. A compound of copper and hæmatoporphyrin (Laidlaw) may be prepared in a similar way. The artificial copper compound appears to be identical with the pigment turacin.

The following is an alternative and in some respects preferable method of preparing these metallic derivatives.

Dilute the alkaline alcoholic solution of hæmatoporphyrin with about twice its volume of glacial acetic acid. Divide this solution into two portions (A and B). Add a trace of copper acetate to A, and a trace of zinc acetate to B, and heat both carefully to the boiling point, or heat both on the water bath for a few minutes, then cool, and examine the solutions with the spectroscope. A shows a 2-banded spectrum somewhat like that of (reduced) alkali-hæmatin; but both bands lie nearer the red end of the spectrum. The α band, which is much the darker, lies a little to the green side of the D-line, and the β band, which is much fainter, lies in the light green (about λ 535-520). Add a drop or two of concentrated hydrochloric acid to A, no obvious change occurs in the absorption spectrum. The copper compound is therefore not readily decomposed by mineral acids. The zinc compound in B shows the spectroscopic characters already described. Add one or two drops of concentrated HCl to B, and examine with the spectroscope. The solution B will now be found to show the absorption spectrum of hæmatoporphyrin in mineral acid solution. The zinc compound is therefore readily decomposed by mineral acids.

[10. Synthesis of hæmatin from hæmatoporphyrin (Laidlaw).]

The following is a rapid method of demonstrating this synthesis:—

Thoroughly mix 8 to 10 drops of blood with 5 c.c. of glacial acetic acid. Heat until the pigment is completely dissolved, cool, and filter. Dilute a small quantity of the filtrate with glacial acetic acid or alcohol, and examine with the spectroscope. The solution, naturally, shows the absorption spectrum of acid oxyhæmatin. Add one or two drops of concentrated HCl to the remainder of the solution, and a small quantity of finely subdivided iron (iron reduced by hydrogen), boil for one or two minutes or heat on the water bath for five minutes. The solution, which acquires a bright red colour, is then filtered. A small quantity when diluted with alcohol or glacial acetic acid shows the

spectrum of acid hæmatoporphyrin. Some care is necessary as regards the duration of heating. If heating be too prolonged the hæmatoporphyrin, which is formed, undergoes further changes, which will be described later, if the heating be too brief, some hæmatin may escape conversion into hæmatoporphyrin.

Add a small quantity of sodium acetate (preferably anhydrous) to the rest of the filtrate, and boil for a minute or two. The bright red solution becomes brown, and a small portion after dilution (if necessary) with glacial acetic acid will be found to show the absorption spectrum of acid hæmatin.

Hæmatoporphyrin, when heated with ferrous acetate, combines with the iron to form oxyhæmatin,—(the reduced) hæmatin, which is probably first formed, being rapidly oxidised to oxyhæmatin by the atmospheric oxygen.

Hæmatoporphyrin + ferrous acetate = oxyhæmatin.

The linkage of hæmatoporphyrin with a salt of iron is completely prevented by free mineral acid. The addition of sodium acetate is therefore essential. The union of hæmatoporphyrin with iron also occurs, when the pigment is heated with Stokes' solution (Laidlaw).

(Reduced) alkaline hæmatin (hæmochromogen) may be prepared from the solution of synthetic acid oxyhæmatin in the following way. Place 5 c.c. of 10 per cent. caustic soda in a test-tube, add at least 10 drops of the acid solution of synthetic (oxy)hæmatin (taking care that the solution is still alkaline by testing with litmus paper), boil, and filter off the precipitate of ferric oxide with some adherent hæmatin. Add a few drops of 10 per cent. sodium hydrosulphite (or a few granules of the solid) to the filtrate. The solution becomes bright red and shows the well-marked absorption spectrum of (reduced) alkali-hæmatin (or hæmochromogen). The synthetic (reduced) hæmatin appears to differ from the natural pigment in not forming a compound with carbon monoxide. There are also certain slight differences in the spectroscopic

characters of the natural and artificial forms of hæmatin, both oxy- and reduced.

[II. Preparation of a stannous derivative of hæmatoporphyrin.]

(1) *Dilute 2 to 3 drops of blood with 5 c.c. of glacial acetic acid, mix thoroughly and heat to the boiling point. The solution shows the absorption spectrum of acid hæmatin.*

Add 3 drops of 2 Molar stannous chloride solution¹ and boil for about one minute. The solution shows the absorption spectrum of acid hæmatoporphyrin. Add a little solid sodium acetate, boil the solution for about a minute, filter and examine with the spectroscope. Two very intense absorption bands will be seen (α , λ 587-570, β , λ 552-530) resembling the bands of oxyhæmoglobin in position and character.

(2) If 2 M. stannous chloride solution is not available, the test may be carried out in the following way:—

Mix one drop of blood with 5 c.c. of glacial acetic acid, heat to the boiling point, add a little solid stannous chloride, a trace of finely granulated tin (preferably precipitated tin), and one drop of concentrated hydrochloric acid. Boil the mixture for about one minute, then add a little solid sodium acetate, again boil for a minute, and filter.

In the presence of free mineral acid (either added to the solution or derived from the stannous chloride) (oxy)hæmatin is converted by the reducing action of stannous chloride into hæmatoporphyrin and ferrous chloride. On adding sufficient sodium acetate to convert all the hydrochloric acid present

¹ Preparation of 2 M. SnCl_2 solution. Place 119 grms. of granulated tin in a resistance glass flask of 500 c.c. capacity, add 300 c.c. of concentrated hydrochloric acid, and heat on a sand bath until the tin has almost completely dissolved, then add 50 c.c. of concentrated hydrochloric acid, heat for a short time, cool, add a little precipitated tin, and make up to 500 c.c. with water.

into acetic acid, the hæmatoporphyrin combines with the tin to form a stannous compound.

Hæmatoporphyrin + stannous acetate = stannous compound of hæmatoporphyrin.

The absorption bands of this pigment are very intense, being recognisable in greater dilution than those of (reduced) alkali-hæmatin. The application of the test to the detection of blood in urine will be described later.¹

[11.] Products of the more prolonged action of powerful reducing agents on hæmatoporphyrin.

Mix 8 to 10 drops of blood with 5 c.c. of glacial acetic, boil until the pigment has dissolved, add a trace of zinc dust, and again boil for about a minute. The solution becomes bright red, and shows the absorption spectrum of "metallic" (zinc) hæmatoporphyrin. If the zinc dust be of good quality, the solution will become light yellow after 5-7 minutes' boiling. If at this stage a small quantity of the solution be decanted into another test-tube, it will be found to darken to a red-brown colour on exposure to air, and will show the spectrum of "metallic" hæmatoporphyrin. On acidification with HCl, it will show the two absorption bands of acid hæmatoporphyrin, and in addition an absorption band in the green and blue (λ 510-475-approximate) closely resembling that of urobilin.

Add 4 to 6 drops of concentrated hydrochloric acid to the original solution, and continue the heating of the fluid on the water bath for about an hour, occasionally adding small quantities of zinc dust, and heating the test-tube over the open flame in order to hasten the reduction process. Not more than 0.2 to 0.3 gm. of zinc dust are required for the reduction. The solution is finally filtered. A portion of the filtrate is diluted with an equal volume of methylated spirit, again filtered, and examined with the spectroscope. It shows a distinct absorption band having the approximate limits (λ 510 to λ 475). This absorption spectrum closely resembles that of hydrobilirubin, and urobilin; but the three pigments differ in their chemical composition.

The reactions which have occurred may be summarised as follows:—

Hæmatoporphyrin \rightarrow Chromogen of hæmatoporphyrin (probably an addition compound of hæmatoporphyrin with hydrogen—Nencki) \rightarrow urobilinoid pigment.

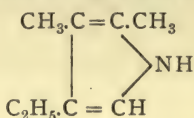
[12.] Formation of a pyrrole derivative by the reduction of hæmatin or hæmatoporphyrin.

Thoroughly mix a small quantity of hæmatin or hæmatoporphyrin with about 20 times as much zinc dust in an agate mortar, transfer

¹ The test may also be applied to the detection of occult blood in fæces.

the mixture to a dry test-tube and heat over the open flame. If a pinewood strip previously dipped in concentrated hydrochloric acid be held in the vapours coming from the test-tube it will be found to become dark red. This reaction which is given by all pyrrole derivatives has already been described.

Nencki obtained a pyrrole derivative known as hæmopyrrole by reducing hæmin with hydriodic acid and phosphonium iodide dissolved in glacial acetic acid. According to Küster, hæmopyrrole is a mixture of three pyrrole derivatives, one of which has the formula :—



Hæmopyrrole in aqueous solution readily undergoes oxidation on exposure to air becoming converted into a pigment spectroscopically resembling urobilin, and like it yielding with ammoniacal zinc hydrate a solution showing distinct green fluorescence. The pyrrole obtained by distilling hæmatin or hæmin with zinc dust possesses similar properties. Similar pyrrole derivatives have been obtained by the reduction of bile pigment (and of chlorophyll). Chemical evidence thus indicates a close relationship between blood, bile, and urinary pigments. For comparison, the products of the catabolism of blood, and the probable sequence of their formation may be summarised thus :—Hæmatin→Hæmatoidin (or bilirubin) + iron compounds (known as “hæmosiderin,”¹ which is probably a mixture of ferric or ferrous oxide with decomposition products of the proteins of the blood) $\xrightarrow{\text{reduction in intestine}}$ Hydrobilirubin→Urobilin and its

Chromogen. Hydrobilirubin contains 9.45 per cent. of nitrogen ; while urobilin only contains 4.11 per cent. (Hopkins and Garrod). The two substances are therefore certainly not identical, as was at one time supposed. For fuller information, the reader is referred to the larger text-books of Physiology, and Physiological and Pathological Chemistry.

PREPARATION OF CRYSTALS OF HÆMIN

(C₃₃H₃₂N₄O₄FeCl—Willstaetter).

(a) Let a drop of blood dry on a slide, cover, and allow a drop of glacial acetic acid to run in under the cover-slip. Heat to the boiling point, and examine with

¹ “Hæmosiderin” is formed when extravasated blood undergoes decomposition in the living organism. For the fate of the iron resulting from the similar disintegration of blood pigment in the liver, the reader is referred to text-books of Physiology.

*the microscope for the dark brown rhombic crystals of hæmin. If these have not appeared, repeat the treatment with glacial acetic acid. In the case of fresh blood, the addition of sodium chloride is not necessary; but, in the case of dried blood stains, it is safer to add a little sodium chloride before treating the stain with glacial acetic acid. Sketch the crystals.*¹

[*(b)* METHOD OF ZEYNEK AND NENCKI.]

Take 10 c.c. of defibrinated blood. Precipitate the proteins by the addition of acetone. Filter off the precipitate and extract it with 10 c.c. of acetone, previously acidified with two or three drops of hydrochloric acid. Place a drop of the dark-brown solution of hæmin so obtained on a slide, cover and examine with the microscope. As the acetone evaporates, minute crystals of hæmin separate out. In order to obtain larger crystals, evaporate the acetone solution of hæmin to about half its bulk, cool and leave in a stoppered vessel overnight. A deposit of hæmin crystals will be found adhering to the bottom and sides of the vessel. Note their bluish-black colour and metallic lustre. On microscopic examination, the hæmin will be found to have separated out in the form of long rhombic needles, largely arranged in rosettes and sheaves. Filter off the precipitate and dissolve in dilute caustic soda or ammonia. The dilute alkali decomposes the hæmin into hæmatin, which dissolves in excess of the alkali, and sodium or ammonium chloride. Add Stokes' solution, or ammonium sulphide, to a small portion of the alkaline solution of hæmatin, and examine spectroscopically = spectrum of hæmochromogen. Acidify the rest of the solution with dilute acetic acid. A voluminous dark-brown precipitate of hæmatin separates out.

¹ If the crystalline deposit be dried by *gentle heat*, and mounted in Canada balsam, a permanent microscopic preparation is obtained.

[(c) SCHALFEJEFF'S METHOD FOR THE PREPARATION OF HÆMIN.]

Heat 20 c.c. of glacial acetic acid, which has previously been saturated with sodium chloride, to 90° - 95°. Then add 4 c.c. of defibrinated blood, and keep the mixture heated for half an hour, at the same time stirring it frequently. Filter the solution while hot into a narrow beaker, and allow it to stand for twenty-four hours. Siphon off the supernatant fluid from the deposit of crystals which has separated out, and wash with water by decantation. The washing may be much facilitated by the use of the centrifuge.

Examine the crystals microscopically, and compare them with those prepared by the previous method, noting the differences in shape and arrangement of the crystals.

EXAMINATION OF BLOOD STAINS (e.g. ON CLOTH).

Cut out the piece of stained cloth and extract with 0.9 per cent. NaCl. Examine a drop of the extract microscopically for red blood corpuscles. Concentrate the remainder of the fluid on the water bath, and test for the formation of hæmin. A still more delicate test for blood so obtained depends upon the preparation of hæmochromogen. Heat the stained piece of cloth with 1 per cent. caustic soda. Cool and filter the solution. Add ammonium sulphide or Stokes' solution to the filtrate, and examine spectroscopically.

An alternative method is to extract the stained cloth with warm glacial acetic acid, and prepare a solution of the stannous compound in the way already described.

[DETECTION OF THE IRON IN HÆMATIN.]

Evaporate an ammoniacal solution of hæmatin to dryness in a porcelain crucible. Incinerate the dry residue.

Add a few drops of pure concentrated HNO_3 and again incinerate. Dissolve the residue in pure dilute HCl . Filter through ash-free filter paper. Add some potassium ferrocyanide to the filtrate. A precipitate of Prussian blue forms.

CHEMICAL EXAMINATION OF HUMAN BLOOD.

The working conditions in a class of practical physiological chemistry make the adequate examination of human blood difficult. The reader is referred to A. E. Wright's "Technique of the Teat and Capillary Glass Tube" (especially chapters i. to v.) for a description of the methods of obtaining and examining samples of human blood. A knowledge of this technique is of great value for students of physiological chemistry.

CHAPTER XIII

MILK

I. GENERAL CHARACTERS

THE milk of all mammals has certain common characters; but its composition varies considerably in different animals. The following description refers mainly to cow's milk, which has been examined more thoroughly than other varieties.

Milk is a bluish or yellowish white opaque fluid which, on microscopic examination (high power), is found to consist of a large number of highly refractive fat globules of varying size suspended in a relatively clear fluid, the milk plasma. When the milk is allowed to stand at room temperature, the fat globules, on account of their lower specific gravity, gradually rise to the surface (at the same time coalescing to form larger particles) as cream. The separation may be hastened by centrifugation. When milk, especially when acid, or cream is subjected to powerful mechanical agitation, as in churning, the fat globules pass from their under-cooled fluid state, at the same time coalescing to form solid macroscopic grains, the mass of fatty material thus obtained being known as butter.

The freezing point (-0.555°) is almost constant. The boiling point is about $+0.2^{\circ}$ above that of water. The reaction of fresh milk varies from P_H 6.6 to 6.8. 100 c.c. of milk require about 7 c.c. of $N/4$ alkali for neutralisation to phenolphthalein. The hydron concentration is raised by boiling. Such milk is only slowly coagulated by rennin. On prolonged heating nearly at the boiling point, a skin consisting essentially of concentrated milk forms on the surface.

On standing, milk becomes sour owing to the conversion of lactose into lactic acid by bacterial action.

Chemical composition.—The following table gives the composition of human and cow's milk. A knowledge of the differences in their composition is important in practical medicine. Milk contains all the food constituents required for the growth and maintenance of the animal organism. The only constituent, which is not present in sufficient quantity, is iron :—

	Cow's Milk (per cent.)	Human Milk (per cent.)
Water	88.0	87.58
Dry Solids	12.0	12.42
Fat	4.8	3.74
Caseinogen	3.0	0.8
Albumin and globulin	0.3	1.21
Total Nitrogen	0.55	0.1847
Extractives—N	0.05	...
Protein—N	0.5	...
Caseinogen—N	0.45	...
Lactose	4.4	6.37
Citric Acid	0.12 to 0.2	...
Urea	0.1	...
Ash	0.7 to 0.8	0.3

Small quantities of the following substances are also present. Lecithin (P_2O_5 of ether soluble material $\times 11.3666$), 0.0499 per cent. (human milk), 0.0629 per cent. (cow's milk), cholesterol and a yellow pigment (traces of both).

The 0.7 to 0.8 per cent. of ash in cow's milk consists of K_2O (0.172 per cent.), Na_2O (0.051), MgO (0.02), CaO (0.198), Fe_2O_3 (0.00035), P_2O_5 (0.182), Cl (0.098), SO_3 (0.021). A large proportion of the phosphoric acid, and almost the whole of the sulphuric acid, are derived from the oxidation of organically bound phosphorus and sulphur.

(a) *Take the REACTION of fresh cow's milk.*

The reaction is neutral or slightly alkaline to litmus. It gradually becomes acid, when the milk is allowed to stand for some time, owing to the lactic acid fermentation of lactose.

(b) *Take the SPECIFIC GRAVITY with urinometer.*

It varies from 1.028 to 1.035.

(c) *Heat some milk in test-tube. No coagulation occurs; but a skin gradually forms on the surface.*

(d) Its OPACITY is due to the presence of fine fat globules.

Shake up a little milk with twice its volume of ether in a test-tube. The opacity is scarcely affected. Add a few drops of caustic soda to a small quantity of milk in a test-tube, then shake with twice its volume of ether. The solution lying subjacent to the ether becomes translucent.

(e) The GUAIAC REACTION succeeds with fresh milk as with blood.

Compare with the behaviour of previously boiled milk.

(f) For the action of the RENNET FERMENT, *see* pp. 111-113.

II. DETECTION AND PARTIAL SEPARATION OF THE CHIEF CONSTITUENTS.

Dilute 1 volume of milk with 3 volumes of water. Then add acetic acid, drop by drop, shaking after each addition of acid, until a flocculent precipitate of CASEINOGEN, with adhering FAT, is thrown down. Filter. To the filtrate add one-fourth its volume of a saturated solution of sodium chloride, and heat. A precipitate of LACT-ALBUMIN and LACTOGLOBULIN is thrown down. Again filter, and neutralise the filtrate with dilute caustic soda. Test a small quantity of the filtrate by Fehling or Trommer = reduction. Test another small portion of the filtrate with Barfoed's reagent = no reduction.

The reduction, in the first case, is due to the presence of LACTOSE. For other tests for lactose, *see* p. 26.

Test the second filtrate also for PHOSPHATES by the following methods:—

(a) *Add to a small quantity of the solution, magnesia mixture = a precipitate of ammonium magnesium phosphate.*

(b) *Acidify with acetic acid, heat the solution, and then add uranium acetate as long as the precipitate which forms continues to increase.*

(c) *Render strongly acid with nitric acid. Then add at least an equal volume of the solution of ammonium molybdate, and heat to 40-50°. A yellow precipitate of ammonium phosphomolybdate separates out.*

The quantity of phosphates is small, and the precipitates which separate from 5-10 c.c. of fluid are therefore slight.

For the separation of fat by Soxhlet's method, *see* p. 348.

III. PRECIPITATION OF CASEINOGEN AS A SALT.

In milk, the caseinogen is in solution in the form of its neutral calcium salt. Free caseinogen is insoluble in water, and the above method of separation is dependent on the fact that acetic acid decomposes the salt into free caseinogen and calcium acetate. It may be precipitated as a salt by half saturation with ammonium sulphate, or by complete saturation with sodium chloride or magnesium sulphate.

1. *To a small quantity of milk add an equal volume of a saturated solution of ammonium sulphate; the caseinogen separates out in the form of a salt along with lactoglobulin. Filter.*

2. *Saturate the filtrate with finely-powdered ammonium sulphate = a precipitate of lactalbumin.*

IV. PREPARATION OF LACTOSE.

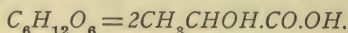
Mix 300 c.c. of milk with about 700 c.c. water, add gradually 30 per cent. acetic acid, shaking thoroughly after each addition, until a flocculent precipitate of caseinogen begins to separate. Allow the solution to stand for a few minutes, then immerse the flask in boiling water, and heat until the proteins have been coagulated. Filter off the precipitate, neutralise the filtrate with calcium carbonate, again filter, and concentrate the filtrate in a porcelain basin to small bulk (about 20 to 25 c.c.) on the water bath. Leave the syrup in the ice-chest until the lactose has crystallised out. The sugar is then freed as far as possible from the mother liquid by pouring off the latter, and partially drying the crystals by pressing them between filter papers. The crystals are then dissolved in about 50 c.c. of boiling water to which a little animal charcoal is added, and the solution filtered. Concentrate the filtrate to small bulk and allow the sugar to crystallise out as before. Then wash the crystals with 80 per cent. alcohol on to hardened filter paper in a Buchner funnel, partially dry by suction with the water pump, and then dry in vacuo over calcium chloride

V. LACTIC ACID FERMENTATION OF MILK. PREPARATION OF OPTICALLY INACTIVE LACTIC ACID.

Dissolve 50 grms. of cane sugar in 500 c.c. of water, add about 30 grms. of calcium carbonate, and 50 c.c. of sour milk, and leave the mixture (in a bottle plugged with cotton wool) for 6 to 8 days

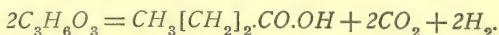
in the incubator at 37°. The mixture should be shaken as frequently as possible.

The greater part of the cane sugar and lactose are hydrolysed and the resulting monosaccharides converted into lactic acid thus—



Transfer the mixture to a flask, boil, filter, concentrate the filtrate to small bulk on the water bath, and leave to crystallise. Filter off the crystalline precipitate in a Buchner funnel, dissolve in as little warm water as possible, add animal charcoal, boil, filter, and allow the purified calcium lactate to separate out as before. Dry the calcium salt thoroughly in vacuo, weigh it, and dissolve in a small quantity of water. Convert the calcium salt into the zinc salt by adding a solution of zinc sulphate containing an equivalent amount of the latter salt (29.4 grms. of crystalline zinc sulphate for 27.5 grms. calcium lactate). Allow the solution to stand for some time, heat slightly, filter off the calcium sulphate, concentrate the filtrate to small bulk and leave in the ice chest for 24 hours. Examine the crystals of zinc lactate microscopically, then filter off the salt, and dry in vacuo. Lactic acid may be obtained from the zinc salt $[Zn(C_3H_5O_3)_2 + 3H_2O]$ by decomposing the latter with H_2S .

Part of the lactic acid is converted into butyric acid, especially if the incubation period be extended beyond 8 days. The latter is easily recognisable by its odour.



GENERAL CONSIDERATIONS ON THE EXAMINATION OF ORGANS.

The organs must be examined as soon as possible after the death of the animal. The animal should be killed by bleeding, in order to obtain the organs as free as possible from blood. In certain cases, it may be also necessary to wash out the vessels of the animal or of the individual organs with .9 per cent. solution of sodium chloride. Adhering connective tissue, blood vessels, nerves, etc., are dissected off, and the organs then finely subdivided by means of a mincing machine. The pulp may then be rubbed up with sand in a mortar. In isolating the cells of glandular organs, it will frequently be found useful to press the elements through muslin previously freed from starch by washing. The greater part of the connective tissue and vessels will be retained by the muslin.

[GENERAL SCHEME FOR THE EXAMINATION
OF ORGANS FOR PROTEINS.]

1. *Extract¹ thoroughly with water. Filter the extract first through calico, and then through filter paper under reduced pressure.*

2. *Extract the residue with .1 to .2 per cent. solution of sodium carbonate, and filter off the alkaline extract.*

3. *Extract the second residue with 5 per cent. magnesium sulphate or sodium chloride, or with 10 per cent. ammonium chloride.*

The FIRST extract may contain the greater part of the *albumins*, some *nucleo-protein*, and *mucins*. It will contain little or no *globulin*.

The SECOND extract may contain *nucleo-proteins* and some *albumin*.

The THIRD extract will chiefly contain *globulins*.

MUSCLE.

[I. PROTEINS OF FRESH MUSCLE WHICH HAS
NOT UNDERGONE RIGOR MORTIS.]

The blood vessels of a rabbit are washed out with a stream of .9 per cent. sodium chloride, injected through the aorta. The muscles are then removed, minced, and extracted for twenty-four hours with 5 per cent. magnesium sulphate. The salt extract is then filtered through well-washed calico.

Perform the following tests with this extract :—

1. *Dilute 1 volume of the extract with 4 volumes of water, and place the solution on the water bath at 40°. A clot of myosin gradually forms. Pour off, and filter the muscle serum. On heating it, a coagulum appears at 63°. If this be filtered off, a further coagulum is obtained at 73°.*

2. *Add a few drops of dilute acetic acid to some of the extract. A precipitate of myosinogen is produced.*

¹ In this, and all similar cases, extraction is greatly accelerated by the use of a bottle-shaker.

3. *On heating the extract, coagula of different proteins may be obtained at 47°, 56°, 63°, and 73°.*

4. *Neutralise the saline extract, and then saturate with magnesium sulphate. The globulins are precipitated. Filter and heat. A coagulum forms at 73° C.*

[II. PROTEINS OF MUSCLE WHICH HAS UNDERGONE RIGOR MORTIS.]

Fresh meat is freed from adhering connective tissue and fat, then finely minced, and extracted with water. The mixture is filtered through calico, and then through paper, with the aid of the filter pump. The residue is then extracted for twenty-four hours with 15 per cent. solution of ammonium chloride.

1. Perform the following tests with the AQUEOUS EXTRACT :—

(a) *The reaction is acid to litmus paper owing to sarcolactic acid (d- α- hydroxypropionic acid). The lactic acid formed by the fermentation of lactose is a racemic mixture of d- and l- lactic acids.*

(b) *On heating, a coagulum is obtained at 55°. The filtrate coagulates at 65°, and the third filtrate at 73°.*

2. SALINE EXTRACT.

(a) *Add a few drops of the saline extract to excess of distilled water = precipitate of myosin.*

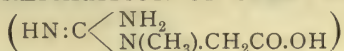
(b) *To some of the solution, add an equal volume of a saturated solution of ammonium sulphate. A precipitate of myosin forms. Filter.*

(c) *Saturate the filtrate with finely powdered ammonium sulphate, heating the solution to about 30° C. Filter. Test the filtrate by the xanthoproteic reaction. A negative result is obtained.*

(d) *Heat some of the extract to the boiling point. Filter, acidify with acetic acid, and add some solution of*

ammonium oxalate. A precipitate of calcium oxalate forms.

PREPARATION OF CREATINE



HYPOXANTHINE, AND SARCOLACTIC ACID FROM MUSCLE.

500 grms. of meat freed as far as possible from fat and tendon are finely minced and extracted with 500 c.c. of water for 15 minutes at 50-60° C., filtered through calico, again extracted with 250 c.c. of water. The united extracts are freed as far as possible from protein by boiling, cooled, and filtered. The filtrate is finally freed from protein and the anions of salts by the addition of basic lead acetate as long as the precipitate continues to increase, care being taken to avoid excess. The fluid is again filtered, and the filtrate freed from lead by sulphuretted hydrogen, which is passed through the warm solution.

The filtrate from lead sulphide is evaporated down to a thin syrup on the water bath (better in vacuo). After being left some time in the exsiccator, crystals of creatine separate out. These are filtered off, washed with dilute spirit (88 per cent. alcohol), and purified by recrystallisation from a small quantity of water. The mother-liquid and water used for washing are united, freed from alcohol on the water bath, and after being cooled are mixed with silver nitrate and excess of ammonia. The precipitate, which forms, is filtered off, and dissolved in hot nitric acid of sp. gr. 1.1 (a little urea may be added to the acid to decompose any nitrous acid). On standing, fine needles of hypoxanthine silver nitrate separate out. A small quantity of the silver compound of xanthine may be precipitated from the acid filtrate by ammonia. The free purine bases may be obtained from their silver compounds by decomposing the latter by suspending them in dilute ammonia and passing through a stream of sulphuretted hydrogen. On evaporating the filtrate from the silver sulphide precipitate one obtains the two bases.

SARCOLACTIC ACID.—The ammoniacal filtrate from the first silver precipitate is just freed from silver by sulphuretted hydrogen, filtered, freed from ammonia by heating on the water bath, and strongly acidified with dilute sulphuric acid. The acid solution is thoroughly extracted with ether, the ethereal extract separated, and the ether distilled off. The residue left after distilling off the ether is diluted with water and neutralised by boiling with zinc carbonate. The solution is then filtered, and the filtrate concentrated on the water bath until crystals appear. The separation of crystals is hastened by the addition of alcohol. The sarcolactate of zinc, which crystallises out, is washed with dilute spirit. It may be purified by recrystallisation from water. The salt crystallises in the form of four-sided prisms, which contain 2 molecules of water of crystallisation (12.9 per cent.). The free acid may be obtained from the salt by dissolving the latter

in boiling water and passing a current of sulphuretted hydrogen through the solution. The filtrate from the zinc sulphide after concentration on the water bath yields a syrup of optically active sarcolactic acid ($[\alpha]_D = +3.5$) :—

PROPERTIES OF SARCOLACTIC ACID AND SOME OF ITS SALTS.—The acid forms a colourless, odourless syrup readily soluble in water, alcohol, and ether. Fermentation lactic acid may be used for the following exercise :—

Test.—5 c.c. of lactic acid are heated with a mixture of 5 c.c. of concentrated sulphuric and 10 c.c. of water in a distillation flask connected with a condenser. The distillate contains acetaldehyde and formic acid, which may be recognised by the usual tests.

SARCOLACTATES.—The zinc salt is lævorotatory ($[\alpha]_D = -6.06$), and contains 29 per cent. of zinc oxide. The calcium salt crystallises in sheaves of very fine needles (4 molecules of water of crystallisation).

CHAPTER XIV

NORMAL CONSTITUENTS OF URINE

GENERAL CHARACTERS

1. Colour.

Note that normal urine is a transparent yellow fluid. When shaken, the froth which forms rapidly disappears on standing.

The intensity of the yellow colour as a rule varies with the concentration, from a light yellow to orange or red-brown. Diabetic urine is an exception to this rule. Pathological pigmentation will be dealt with later.

2. Transparency.

When acid, the fresh urine is clear. A finely flocculent precipitate or nubecula, consisting of mucin secreted by the epithelium of the bladder, gradually separates out after the urine has stood for some time. If an acid urine be concentrated, a yellow or red precipitate may separate out when the urine cools to room temperature. This sediment, sometimes known as sedimentum lateritium, consists of acid sodium urate with adherent pigment. It dissolves readily when the urine is heated.

An alkaline or neutral urine may be distinctly turbid either when fresh or after standing owing to the separation of earthy phosphates, calcium oxalate, and in some cases calcium carbonate. This precipitate does not disappear, but may rather be increased in amount on heating. It dissolves readily on the addition of dilute acetic, hydrochloric, or nitric acid.

In pathological conditions the urine may be highly turbid owing to the presence of excess of mucin or formed constituents such as pus, epithelial cells, blood, fat globules, casts of various kinds, or bacteria. The formed elements may be separated by centrifugation, and examined microscopically.

3. *Dip red and blue litmus paper into the urine.*

The REACTION of fresh human urine is acid to litmus paper. The acidity is not due to free acid, but to acid phosphates. Variations in reaction are mainly dependent upon the nature of the diet, digestion, and the amount of water excreted. If urine be allowed to stand in a warm place, ammoniacal fermentation takes place, the urea being converted into ammonium carbonate by the action of the *Micrococcus ureæ*. The acidity of the urine is lowered during gastric digestion ("alkaline tide"), raised during fasting, and all conditions which interfere with the excretion of CO_2 in the expired air, *e.g.* during sleep (Leathes).

4. Specific gravity.

Take the SPECIFIC GRAVITY with a urinometer.

It is extremely variable. The average is from 1.017 to 1.020. The urinometers are graduated for a fixed temperature. When used for taking the specific gravity of a urine, at a temperature above or below the standard temperature, the following correction has to be made:—For each 3° above the standard temperature, .001 is to be added to the reading of the urinometer; while for each 3° below the standard temperature, .001 is to be subtracted from the reading. If, for example, a urinometer, graduated at 15°C. , indicates a specific gravity of 1.016 in a urine, the temperature of which is 21°C. , the specific gravity of the urine at 15°C. would be $1.016 + .002 = 1.018$.

The specific gravity may also be taken with the pyknometer (see p. 293).

5. Volume.

The volume varies with the amount of liquid taken with the food, and with the excretion of water by other channels than the kidneys. It is generally largely increased in diabetes, and diminished in febrile conditions. Average amount for adult, 1500 c.c.

6. Optical Characters.

Normal urine is almost invariably lævorotatory. The rotation is usually so small, varying from $.01^{\circ}$ to $.04^{\circ}$, that polarimetric estimations of sugar in cases of glycosuria are not appreciably affected.

7. The Freezing Point

of normal urine is about -1.7° below that of water. The approximate limit of variation is from -0.08° to -3.5° . As a rule, any value numerically less than -1.2° is pathological.

8. The Characteristic Odour

of fresh normal urine is due to unidentified constituents.

CHIEF NORMAL CHEMICAL CONSTITUENTS.

Composition of normal urine (Folin). **Effect of diet.** The results given in columns (1) represent the composition of the urine eliminated in twenty-four hours by an adult male taking a standard diet consisting of 119 grms. protein, 148 grms. fat, and 225 grms. carbohydrates. This daily ration contained 19 grms. nitrogen, 6.13 grms. Cl_2 , 3.7 grms. SO_3 , and 5.7 grms. P_2O_5 . The values in (2) give the composition of urine from the same individual on a diet poor in protein, containing 400 grms. partially digested starch, 300 c.c. of cream (containing 15-20 per cent. of fat) and 6 grms. of sodium chloride diluted with 1500 c.c. water.

	Initial body weight, 86.7 kilos. (1)			Weight on reduced diet, 85.7 kilos. (2)		
	Vol. of urine, 1170 c.c. Sp. gr., 1.028.			Vol. of urine, 385 c.c. Sp. gr., 1.027.		
	Grms.	N ₂ in grms.	In % of total N ₂ .	Grms.	N ₂ in grms.	In % of total N ₂ .
Total N ₂ . . .	—	16.8	100	—	3.6	100
Urea	32.1	14.7	87.5	4.7	2.2	61.7
NH ₃ (c.c. N/10) . . .	(351)	0.49	3.0	(299)	0.42	11.3
Uric acid	0.53	0.18	1.05	0.26	0.09	2.5
Creatinine	1.58	0.58	3.6	1.60	0.60	17.2
Undetermined N . . .	—	0.85	4.85	—	0.27	7.3

OTHER CONSTITUENTS.

	(1)			(2)	
		In % of total sulphur.		In % of total sulphur.	
Total acidity in c.c. N/10 . . .	805		224		
Total P ₂ O ₅ in grms.	4.1		1.0		
Chlorine in grms.	6.1		1.6		
Total S as SO ₃	3.64	100	0.76	100	
Inorganic SO ₃	3.27	90	0.46	60.5	
Ethereal SO ₃	0.19	5.2	0.10	13.2	
Neutral S as SO ₃	0.18	4.8	0.20	26.5	

I. INORGANIC CONSTITUENTS.

1. Chlorides.

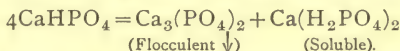
To a small quantity of urine add silver nitrate; a precipitate of silver chloride and phosphate forms. Acidify with nitric acid; the silver phosphate dissolves, while the silver chloride remains undissolved. The latter also dissolves in ammonia.

Amount varies with the quantity of chlorides in the food. It is much diminished in febrile conditions and during the formation of pathological exudates. Average daily amount excreted by an adult, about 15 grms.

2. Phosphates

are present, as acid phosphates of the alkalies, and of calcium and magnesium. Orthophosphoric acid is tribasic, and therefore yields three classes of salts by the replacement of one, two, or three hydrogen atoms—dihydrogen phosphates (MH_2PO_4), monohydrogen phosphates (M_2HPO_4), and tribasic phosphates (M_3PO_4). When M is represented by Na or K, all three classes are soluble in water. The tribasic salts undergo hydrolytic dissociation when dissolved in water, yielding monohydrogen phosphates and free alkali. They are only stable in presence of excess of alkali.

The dihydrogen phosphates of calcium [$\text{Ca}(\text{H}_2\text{PO}_4)_2$] and magnesium are both soluble in water. The monohydrogen phosphates of calcium and magnesium (MgHPO_4) are only sparingly soluble in water. The former is much less soluble than the latter, and is sometimes found in urinary sediments as prismatic crystals ($\text{CaHPO}_4 + 2\text{H}_2\text{O}$). MgHPO_4 , on account of its greater solubility, is rarely, if ever, found in urinary deposits. On boiling, CaHPO_4 decomposes thus:—



$\text{Ca}_3(\text{PO}_4)_2$ and $\text{Mg}_3(\text{PO}_4)_2$ are both insoluble in water, and separate as flocculent precipitates when caustic alkali is added to the urine. If ammonia be added to the urine, part of the phosphates separates as NH_4MgPO_4 . Phosphates of calcium and magnesium, containing a higher percentage of base than the tribasic, have also been obtained.

The composition of the phosphates of the urine varies according to the reaction, and the relative amounts and character of the basic radicles and phosphoric acid radicles present (see chapter on "Reaction"). The quantity of phosphoric acid in the urine is always more than sufficient to unite with all the calcium and magnesium.

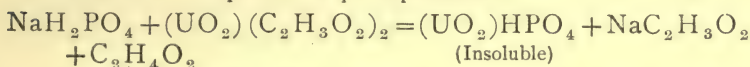
About 6 per cent. of the total phosphorus of the urine is present in organic combination.

(1) (a) *Render a small quantity of urine alkaline by the addition of caustic soda, and heat gently. A precipitate of calcium ($\text{Ca}_3(\text{PO}_4)_2$) and magnesium phosphates ($\text{Mg}_3(\text{PO}_4)_2$) forms. Filter.*

(b) *The filtrate contains the soluble alkaline phosphates. Add magnesium mixture to the filtrate; the soluble phosphates are precipitated in the form of ammonio-magnesium phosphate (triple phosphate) having the formula, NH_4MgPO_4 .*

2. Both alkaline and earthy phosphates may be precipitated by uranium acetate.

Render a small quantity of the urine distinctly acid with an acetic acid solution containing sodium acetate, and then add a solution of uranium acetate as long as a precipitate continues to form. Heat the fluid to about 80°C . in order to complete the precipitation.

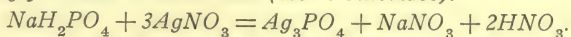


3. Both alkaline and earthy phosphates may also be precipitated by an acid solution of ammonium molybdate.

Render a small quantity of urine strongly acid by the addition of nitric acid, then add excess of acid ammonium molybdate solution, and heat to about 50°C . The fluid first becomes yellow. Later, a precipitate of ammonium-phospho-molybdate separates out.

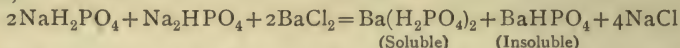
Average daily amount of P_2O_5 excreted is 2.5 grms. The quantity varies mainly with the amount of phosphates in the food. In part, it also varies with the quantity of organically combined phosphorus in the diet, and the extent of decomposition of substances containing phosphorus within the organism.

[4. *Silver nitrate solution gives a light yellow precipitate of silver phosphate, which is readily soluble in nitric acid, but only very sparingly soluble in acetic acid (see 1. Chlorides).*



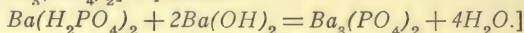
Since nitric acid is set free by this reaction, the precipitation is incomplete. The addition of sodium acetate renders precipitation complete.]

[5. *Detection of dihydrogen phosphates in the urine.* When a solution of barium chloride is added to a solution, such as urine, containing both mono- and di-hydrogen phosphates, the former only are precipitated thus,



If the precipitate be filtered off, any phosphoric acid in the filtrate represents the dihydrogen phosphates. Upon these facts has been based a method for determining the relative amounts of mono- and di-hydrogen phosphates in the urine (Freund).

Add 1 c.c. of 10 per cent. barium chloride solution to 5 c.c. of urine, shake thoroughly, and filter through close filter paper, re-filtering if necessary until a clear filtrate is obtained. Add barium hydrate solution to the filtrate; a precipitate of tribasic phosphate [mainly $\text{Ba}_3(\text{PO}_4)_2$] separates.



3. Sulphates.

1. *To some urine add baryta mixture, as long as a precipitate continues to form. The precipitate consists of barium phosphate and sulphate. Acidify with hydrochloric acid. The barium sulphate remains undissolved, while the barium phosphate dissolves.*

This precipitate contains the whole of the inorganic sulphates. The sulphuric acid of the urine also exists in the form of ethereal compounds, with phenol, indoxyl, and other aromatic substances. These compounds form soluble barium salts.

2. *For the detection of the ethereal sulphate, filter off the precipitate of barium sulphate, and boil the acid filtrate. The solution clouds slightly on boiling, due to the decomposition of the ethereal sulphates into barium sulphate, and the aromatic substances with which the sulphuric acid was previously in ethereal combination.*

3. *Slightly acidify a small quantity of urine (about 2 c.c.) with dilute hydrochloric acid, and add about 8 c.c. of a solution of benzdine hydrochloride.¹ A white crystalline precipitate of benzdine sulphate rapidly separates out.*

The average quantity of sulphuric acid excreted in the

¹ The strength of this solution (Drummond and Rosenheim) is given in the quantitative section of this book.

form of sulphates during the twenty-four hours is 2.5 grms. The sulphuric acid of the urine arises only to a very slight extent from the sulphates of the food. By far the greater proportion is derived from the decomposition of proteins within the organism. The quantity excreted varies directly with the excretion of nitrogen. The relation $\frac{N}{H_2SO_4}$ has the fairly constant value 5.

The chief metals of the urine are sodium, potassium, ammonium, calcium, magnesium, and iron.

[4. Sodium.]

Evaporate to dryness about 20 c.c. of urine. Introduce, by means of a loop of clean platinum wire, a small quantity of the crystalline residue into a Bunsen flame. Note the yellow colour of the flame, and, on spectroscopic examination, the bright yellow sodium line.

The quantity of sodium excreted varies from 4 to 7 grms. in the twenty-four hours' urine.

[5. Potassium.]

Evaporate over the open flame 100 c.c. of urine to about one-eighth of its bulk. Allow the solution to cool, then filter. To the filtrate add a little concentrated solution of tartaric acid, or better, of sodium hydrogen tartrate. Mix the fluids well. On standing overnight in a cool place, crystals of acid potassium tartrate separate out.¹

The quantity of potassium, reckoned as K_2O , excreted in the twenty-four hours, varies from 2 to 4 grms. The quantity of sodium and potassium excreted is largely dependent on their amounts in the food.

6. For the detection of **ammonia**, see p. 310.

¹ Acid ammonium tartrate may also be present. The two salts may be distinguished from one another by the fact that on complete incineration the potassium salt leaves an alkaline residue, while the ammonium salt is volatilised.

[7. Calcium.]

Render 200 c.c. of urine strongly alkaline with ammonia, then heat. After a few minutes filter off the precipitate of earthy phosphates. Dissolve the precipitate in dilute acetic acid. To the clear filtrate add ammonium oxalate, and heat gently. A precipitate of calcium oxalate forms. Examine part of the precipitate with the microscope.

[8. Magnesium.]

Filter off the precipitate of calcium oxalate, and render the filtrate alkaline with ammonia. A precipitate of ammonium magnesium phosphate forms on standing.

The average amount of calcium, reckoned as calcium oxide, excreted in the twenty-four hours is .160 grm.; that of magnesia, about .23 grm.

9. The iron in the urine appears to exist only in organic combination, and the quantity is extremely small. It can only be detected in normal urine, after evaporation and incineration of the residue or by Neumann's method (p. 254).

II. ORGANIC CONSTITUENTS.

1. Urea $\left(\text{CO} \begin{Bmatrix} \text{NH}_2 \\ \text{NH}_2 \end{Bmatrix}\right)^1$

By far the most important of the organic constituents is urea.

[1. PREPARATION OF UREA NITRATE.]

Place about 50 c.c. of urine in a porcelain basin on the water bath, and evaporate down to about one-fifth of its original bulk. Cool the concentrated urine.² Filter. Place a drop of the concentrated urine on a slide. Mix with a drop of pure concentrated nitric acid, and examine under the microscope. Thin plate-like crystals of urea nitrate separate out. To the remainder of the concen-

¹ According to Werner, this formula does not represent the true structure of urea ("Journal Chem. Soc.," vol. 109, p. 1120).

² This is best done by finally floating the basin on ice water.

trated urine add an equal volume of pure concentrated nitric acid. Keep the mixture cool during the addition. Glistening plate-like crystals of urea nitrate separate out. Pour off the excess of fluid and dry the crystals on a porous porcelain plate. Suspend the crystals in 20 to 30 c.c. of 70 per cent. alcohol and gradually add solid barium carbonate, shaking thoroughly after each addition, until effervescence of carbon dioxide has ceased, and the barium carbonate is present in excess. Heat on the water bath, filter, again boil the filtrate on the water bath with a little animal charcoal, filter, concentrate the filtrate to dryness on the water bath and extract the urea from the residue with absolute ethyl alcohol or acetone.

Urea may be more easily obtained by evaporating about 100 c.c. of urine to dryness on the water bath, and extracting the residue at least twice with successive portions of 96 per cent. alcohol (about 20 c.c. in all), or with 30 c.c. of acetone. Decant the solution of urea in alcohol or acetone, filter into a porcelain basin, and evaporate on the water bath. Urea crystallises out. Extraction with acetone yields purer urea less contaminated with salts and pigments than that obtained by extraction with alcohol.

For the following tests use pure dry urea.

2. SOLUBILITIES.

Urea dissolves readily in an equal weight of water to form a neutral solution. It dissolves less readily in cold absolute ethyl alcohol (about 1 in 5). Boiling alcohol dissolves about an equal weight of urea. Urea is also soluble in methyl, butyl, and amyl alcohols, and in acetone.

Dissolve with the aid of heat a little urea in a few drops of ethyl alcohol. Place a drop of the solution on a slide, and examine microscopically the crystals of urea which separate out in the form of four-sided prisms.

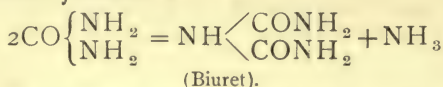
Urea is insoluble in pure ether (free from water and alcohol) and in chloroform.

3. *Dissolve a few crystals of urea in a few drops of water. Add an equal volume of a saturated solution of oxalic acid. Crystals of urea oxalate separate out as*

thick rhombic plates. Place a small quantity on a slide, cover, and examine microscopically.

4. To a concentrated aqueous solution of urea add an equal volume of pure nitric acid, keeping the solution cool during the addition of the acid. A copious deposit of crystals of urea nitrate separates out. The crystals have the form of irregular hexagonal plates.

5. Place a small quantity of urea in a dry test-tube. On heating, the urea melts and gives off ammonia, which may be detected by the usual methods. A sublimate of ammonium carbonate¹ is deposited on the sides of the test-tube. Continue the heating until the melted mass commences to solidify. By heating urea above its melting point biuret is formed.



Cool the residue and dissolve in dilute caustic soda. Then add a few drops of very dilute cupric sulphate solution = a pink solution.

[6. MELTING POINT DETERMINATION.]

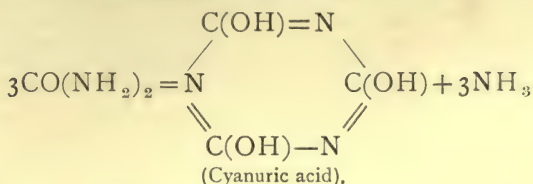
Take a small sample of finely powdered urea, previously dried in vacuo at room temperature. Place it in a watch glass. Then scoop up a little of the urea with the open end of a small capillary tube (about $2\frac{1}{2}$ in. long, and closed at one end). The urea can then be displaced to the bottom of the tube by tapping the closed end on the bench. The urea should form a column of about 4 mm. length at the bottom of the tube. The tube is then attached to a thermometer either by simply moistening the bulb of the thermometer with sulphuric acid, or by means of a rubber band, and the melting point taken in a sulphuric acid bath in the manner already described.

Urea melts at 130° to 132° C.

[7. Again, heat a small quantity of urea in a dry test-

¹ The sublimate is mainly cyanuric acid, if the urea be pure and previously dried in the exsiccator.

tube. Continue the heating until the mass has completely solidified. Then cool and dissolve the residue in dilute caustic soda. On acidifying the alkaline solution by the gradual addition of dilute hydrochloric acid, a precipitate of cyanuric acid is thrown down.]



[8. Heat a small quantity of urea on a piece of platinum foil. It first melts, giving off ammonia, then solidifies to form cyanuric acid, and ultimately volatilises completely, leaving no residue. On heating, each molecule of cyanuric acid breaks up into three molecules of cyanic acid.]



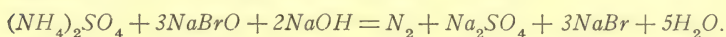
9. To a dilute aqueous solution of urea add gradually some aqueous solution of mercuric nitrate. A white precipitate of variable composition, containing urea in combination with mercuric nitrate, separates out. The precipitate is soluble in sodium chloride solution.

10. To a small quantity of an aqueous solution of urea add some solution of sodium hypobromite; bubbles of nitrogen are evolved. The carbon dioxide which is also formed is fixed by the alkali present.



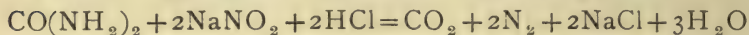
Ammonium salts are decomposed in a similar way.

Add sodium hypobromite solution to a solution of ammonium sulphate. Note the effervescence which occurs.



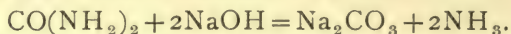
11. To a small quantity of an aqueous solution of urea add a few drops of a saturated aqueous solution of sodium nitrite, and acidify with dilute hydrochloric acid or 30 per cent. acetic acid. Bubbles of nitrogen and carbon

dioxide are evolved. The presence of the latter gas may be demonstrated if the test-tube be fitted with a bored stopper, through which a bent glass tube is passed. By means of the glass tube the gases are passed over baryta water. A precipitate of barium carbonate forms.



All substances containing an amine group are decomposed in a similar way (see page 143), and usually more rapidly than urea.

12. Heat a few crystals of urea with aqueous caustic soda (preferably about 30 per cent.). Ammonia is evolved and sodium carbonate formed.

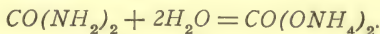


Vigorous boiling is essential, since the decomposition takes place slowly.

[13. Urea forms compounds with aldehydes. The most important of these compounds is that with furfural. Add four drops of concentrated hydrochloric acid to 2 c.c. of a freshly prepared concentrated aqueous solution of furfural. If the furfural have not undergone oxidation, the mixture should not acquire a red colour on standing. Dissolve a crystal of urea in the acid furfural solution. In a few minutes the solution acquires a deep violet colour.]

[14. UREASE REACTION.]

Add a small quantity of a solution of the enzyme urease (an active extract of soya bean) to 5 c.c. of urine previously diluted with 4 volumes of water in a tube labelled (A). Add to another 5 c.c. portion of diluted urine (B), previously boiled and cooled extract of soya bean. Place both test-tubes in the water bath at 35°-40° for 15 minutes, then add a few drops of litmus solution to each and compare the colours. The fluid in (A) has become distinctly alkaline owing to the conversion of the neutral substance—urea into ammonium carbonate, while the fluid in (B) gives the same colour with litmus as the original urine.



The quantity of urea excreted varies with the amount of protein decomposition in the organism. The average amount excreted by an adult in twenty-four hours is about 30 grms.

2. Uric Acid ($C_5H_4N_4O_3$).

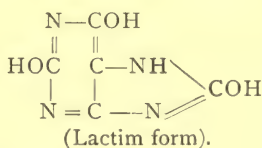
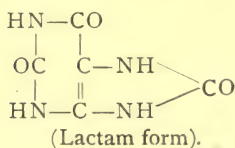
[1. PREPARATION FROM HUMAN URINE.]

Mix 100 c.c. urine with 5 c.c. of concentrated hydrochloric acid, and allow the mixture to stand for twenty-four hours. A dark red-brown deposit will be found to have separated out on the bottom and sides of the vessel, some also frequently floating on the surface. Filter some of the solution. Place some of the deposit on a slide, cover, and examine microscopically. Note highly pigmented crystals of uric acid of great variety of form. The fundamental form is rhombic or whetstone. The uric acid may be purified by repeated solution in dilute caustic alkali and precipitation with hydrochloric acid.

[2. PREPARATION FROM SERPENT'S URINE.]

Dissolve a small quantity of serpent's urine, with the aid of heat, in 5 per cent. caustic soda solution. Note that ammonia is given off. Cool and filter the solution. Pour the alkaline solution into excess of hot dilute hydrochloric acid (1 in 5). A copious precipitate of uric acid separates out. Cool the solution, filter off the precipitate, and wash with water. Examine a small quantity of the deposit microscopically. Note the small colourless transparent rhombic plates of uric acid.

3. Uric acid exists in two tautomeric forms, as represented by the following formulæ:—



4. SALTS OF URIC ACID.

Uric acid is a dibasic acid, forming neutral, *e.g.* sodium urate, $C_5N_4O_3H_2Na_2$, and acid salts ($C_5N_4O_3H_8Na$).

The dibasic salts only exist in presence of excess of alkali, being dissociated by water into monobasic urates, usually known as bi- or di-

urates and free alkali (hydrolytic dissociation). Carbon dioxide decomposes solutions of the dibasic salts into monobasic salts, and carbonate of the alkali. Disodium hydrogen phosphate reacts with uric acid to form monosodium dihydrogen phosphate and monosodium urate.

The monobasic salts exist in two tautomeric forms corresponding to the two modifications of uric acid. The diurates derived from the lactam form are unstable and in solution gradually undergo conversion into the more stable and less soluble lactim form (Gudzent). The following are the solubilities of the chief diurates in cold and boiling water :— NaHU (1 in 1150, 1 in 122), KHU (1 in 790, 1 in 75), LiHU (1 in 370, and 1 in 39), $\text{NH}_4\cdot\text{HU}$ (1 in 1600), CaH_2U_2 (1 in 603, 1 in 276), BaH_2U_2 (1 in 7900, 1 in 2700). U represents the uric acid radicle. The ammonium salt is quite insoluble in saturated solutions of ammonium chloride (Hopkins).

The so-called quadriurates or compounds of one molecule of diurate with a molecule of uric acid are probably mixtures rather than true compounds (Tunnicliffe and Rosenheim).

Pure uric acid, prepared from serpent's urine, is to be used for the following tests :—

5. SOLUBILITIES.—Note that the acid is almost insoluble in water (1 in 16,000 parts). In boiling water it is relatively much more soluble (1 in 1600).

(a) *Heat a small quantity of uric acid with excess of water. Cool. Test the solution with a slip of blue litmus paper.*

No reddening of the paper is produced, owing to the extremely slight solubility of the acid.

(b) It is insoluble in alcohol and ether.

(c) *Dissolve a little finely powdered uric acid in concentrated sulphuric acid, aiding the solution by heating to 40° C. on the water bath. Cool, and then pour the sulphuric acid solution into large excess of distilled water (about 20 volumes). Uric acid is precipitated in the form of fine microscopic rhombic crystals.*

(d) *Uric acid dissolves readily in caustic alkalies and alkaline carbonates, to form the neutral urates. Test its solubility in 5 per cent. caustic soda.*

[(e) *Certain organic bases, e.g. diethylenediamine (piperazine) and methylglyoxalidine (lysidine) form readily soluble salts with uric acid. Place a little uric acid on a slide, cover, and allow a drop or two of 10 per cent. piperazine solution to flow under the cover-slip. Examine microscopically. The uric acid crystals rapidly dissolve.*]

6. URIC ACID AS A REDUCING AGENT.—In alkaline solution, uric acid is a reducing agent.

(a) *Dissolve a small quantity of uric acid in sodium carbonate solution. Add ammoniacal silver hydrate to part of the alkaline solution. A black precipitate of metallic silver separates out. Another method of performing the same reaction is to allow a drop of the alkaline urate solution to fall on a slip of filter paper moistened with silver nitrate. A black stain, due to deposition of metallic silver, is produced on the filter paper.*

(b) *Dissolve a little uric acid in 5 per cent. caustic soda. Add some Fehling's solution, and heat to the boiling point. If the urate be in excess, a white precipitate of cuprous urate separates out. Part of the uric acid is oxidised, and reduces the cupric hydrate to cuprous hydrate. The latter unites with some unchanged urate to form insoluble cuprous urate. Add more Fehling's solution and continue to heat. Red cuprous oxide begins to separate out along with the cuprous urate.*

(c) *Uric acid dissolved in dilute sulphuric acid reduces potassium permanganate (see p. 324).*

[(d) FOLIN'S REACTION.]

Mix 1 or 2 c.c. of the reagent¹ with a trace of uric acid suspended in 1 c.c. of water, then add excess of saturated sodium carbonate solution

¹ To 750 c.c. water add 100 grms. of sodium tungstate and 80 c.c. of 85 per cent. phosphoric acid (H_3PO_4). Boil gently for two hours in a flask with reflux condenser, cool, and dilute to one litre. Two c.c. of this solution give the maximal colour obtainable with 1 mgm. of uric acid.

(3 to 10 c.c.). The solution becomes blue. The test is positive with solutions containing 1 part of uric acid in 500,000 parts of water.

(e) Detection of uric acid in urine and other solutions (Folin-Macallum).

Place 2 to 5 c.c. of urine in a porcelain basin, add one drop of saturated oxalic acid solution, and evaporate to complete dryness on the water bath. Cool, extract with 10 to 15 c.c. absolute alcohol (or preferably a mixture of 2 volumes of anhydrous ether and one volume of methyl alcohol), and allow to stand for a few minutes to remove polyhydricphenols which also give the colour reaction with the phosphotungstate reagent. Carefully decant the alcohol, and add 10 c.c. of water and one drop of a saturated solution of sodium carbonate to the residue. Stir to ensure complete solution of the uric acid and transfer to a test-tube or beaker, add 1 c.c. of the reagent and 20 c.c. of saturated sodium carbonate solution. The solution becomes blue owing to the presence of uric acid.

7. Dissolve a little uric acid in dilute caustic soda. Add a small quantity of magnesia mixture and a few drops of silver nitrate solution. A gelatinous precipitate of silver magnesium urate forms.

8. MUREXIDE REACTION.

Place a very small quantity of uric acid on a piece of glazed porcelain, e.g. the lid of a crucible, add two drops of concentrated nitric acid, and carefully evaporate to dryness over the open flame. Allow the yellowish-red residue to cool, then add a drop of ammonia to one side of the residue, and a drop of caustic potash solution to the other. A purple colour, due to the formation of ammonium purpurate, is developed in the one case, a bluish-violet, due to formation of the potassium salt, in the other.

9. Some additional colour reactions for the detection of uric acid and other purine derivatives (unpublished results).

(1) Add 1 c.c. of dilute nitric acid (1 vol. of nitric acid of sp. gr. 1.42 diluted with 9 vols. water) to 0.01 gm. of uric acid placed in a test-tube. Immerse the tube in boiling water for 6 to 8 minutes, cool thoroughly under the tap, dilute with 5 c.c. of water, neutralise by the gradual addition of calcium carbonate, waiting after each addition until effervescence has ceased, shake thoroughly, allow to stand for a few minutes shaking frequently, and filter from excess

of calcium carbonate. Add about 5 c.c. of a saturated solution of sodium acetate to the filtrate, and then add gradually a freshly prepared 10 per cent. solution of ferrous sulphate or ferrous ammonium sulphate, until the dark blue colour, which develops, has reached its maximal intensity.

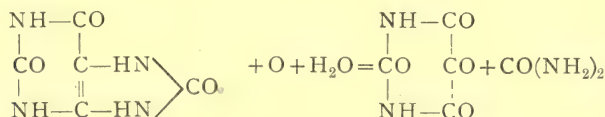
Under the prescribed conditions, this reaction is negative with guanine, xanthine, caffeine, hypoxanthine, and adenine, uric acid being the only purine derivative which gives a positive reaction.

(2) Add 2 c.c. of bromine water to .01 gm. of uric acid or 2.5 c.c. of bromine water to .01 gm. of the other purine derivatives already mentioned, shake up, and allow to stand for 15 to 30 minutes in the cold. The bromine solution is rapidly partially decolorised by uric acid, xanthine, guanine, and caffeine, and little affected by hypoxanthine and adenine. Immerse the tubes in boiling water until all the excess of bromine has been driven off, and the solutions have become perfectly colourless. Cool thoroughly under the tap, and add an equal volume of a saturated solution of sodium acetate (or alternatively dissolve some crystals of sodium acetate in the solutions), then add a 10 per cent. solution of a ferrous salt (freshly prepared) until the maximal depth of colour is obtained. A deep blue colour is obtained with uric acid, guanine, xanthine, and caffeine, a relatively faint blue colour with hypoxanthine and a negative result with adenine. On adding sodium acetate to the adenine solution after oxidation with bromine water a white flocculent precipitate (probably unchanged adenine) separates.

In carrying out the bromine water reaction with unknown quantities of uric acid or other purine derivatives, add the bromine water drop by drop as long as decolorisation occurs fairly rapidly in the cold, then add two or three additional drops of bromine water, heat on the water bath, and afterwards follow the procedure already described.

EXPLANATORY NOTES.

(a) When uric acid is oxidised by acid oxidising agents (such as HNO_3 , Br_2 , Cl_2 , potassium chlorate and HCl , and others), it yields alloxan (mesoxalylurea) and urea, thus :—

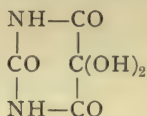


Other purine derivatives offer a greater resistance to oxidation. The resistance to oxidation also varies with the nature of the purine base.

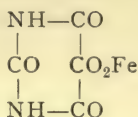
Caffeine yields dimethyl alloxan instead of the alloxan yielded by most of the other purine derivatives.

Alloxan separates from a cold aqueous solution with four molecules of water of crystallisation, and with one molecule of water of crystallisation

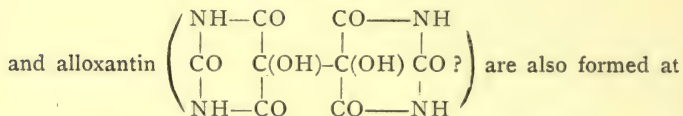
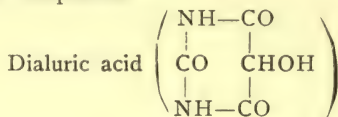
from a warm aqueous solution. The last molecule of water is only driven off at 150° – 160° , and its removal probably involves partial decomposition of alloxan. It may therefore be regarded as an integral part of the molecule, and the formula of alloxan may therefore be written—



Alloxan forms a dark blue compound with ferrous salts (Wöhler and Liebig) having the formula—



The foregoing tests are based on this fact. Mono- and di-methylalloxans form similar compounds.



an earlier stage of the process of oxidation. Both can readily be obtained by the reduction of alloxan.

Alloxantin also gives a blue colour with ferrous salts. The writer has not yet ascertained whether the colour reaction is also obtained with dialuric acid.

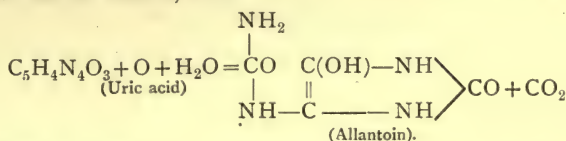
(b) If the oxidation be too prolonged, the colour reaction becomes gradually fainter as alloxan is oxidised to parabanic acid (oxalylurea) and carbon dioxide, and still later is converted into oxalic acid and urea. This fact does not appreciably interfere with the first test, since the colour reaction is still obtainable after .01 grm. of uric acid has been heated with 1 c.c. of dilute nitric acid (1 in 10) for 35 minutes. The maximal colour reaction, however, is obtained when uric acid has been heated for 6 to 8 minutes with dilute nitric acid.

(c) The sodium acetate is added as "a buffer substance" to establish a suitable hydron concentration for the formation of the ferrous compound of alloxan. (The dissociation constant of alloxan is $2.32 \cdot 10^{-7}$ —Wood.) Ammonia may also be used, but has the disadvantages of producing a

precipitate of ferrous hydrate, and of displacing the iron from combination with alloxan, when added in excess.

(d) The delicacy of this colour reaction has not yet been accurately determined, but appears to be much greater than that of the murexide test. Alloxan also forms coloured compounds with amino acids, and its oxime (violuric acid) forms coloured salts with many bases. Practical details with regard to these reactions are omitted here, since the colours yielded are much less intense than that with ferrous salts.¹

10. Products of the oxidation of urates in alkaline solution. One mol of a urate, when oxidised with .66 mol of potassium permanganate, yields allantoin and carbon dioxide, thus :—



Preparation of allantoin. Suspend 1 grm. of uric acid in 25 c.c. water, and add caustic soda solution (1 in 10) drop by drop until all the uric acid has dissolved as sodium urate. Add 10 c.c. of a solution of potassium permanganate (6.2 per cent.), mix thoroughly, and allow to stand for one hour. Then filter off the precipitate of manganese dioxide, acidify with acetic acid, and concentrate the filtrate in a porcelain basin on the water bath to about 5 c.c. The allantoin, which crystallises out, may be purified by recrystallisation from water.

(a) Heat a small quantity of allantoin in a dry test-tube. It chars and melts with decomposition at about 234°. A white sublimate is deposited on the sides of the tube, and a distinct odour of hydrocyanic acid, and later of ammonia, can be detected.

(b) Dissolve 0.3 grm. of allantoin in 30 c.c. of water, heating the fluid on the water bath until solution is complete (solubility in water 1 in 160 at 20°, about 1 in 30 at 100°). Note that the solution is practically neutral to litmus (very faintly acid).

(c) Allantoin dissolves very readily in aqueous alkalies, is only sparingly soluble in ethyl and amyl alcohols, and is insoluble in ether.

(d) Concentrate a few drops of the aqueous solution on a microscopic slide, heating gently over a bunsen flame, set aside to cool, and examine the crystals microscopically (fairly large prismatic needles of the monoclinic system frequently massed in spherical clumps).

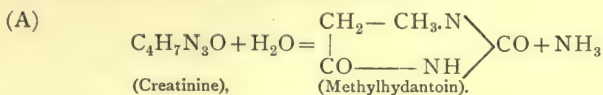
(e) Add a drop or two of dilute mercuric chloride (0.5 to 1 per cent.) to 1 c.c. of the aqueous solution of allantoin, and the same amount of dilute mercuric nitrate to another portion. No precipitation occurs in the first case, but precipitation occurs in the second case.

Then add gradually dilute caustic soda solution to each solution. Result, a white precipitate soluble in excess of alkali. On heating the

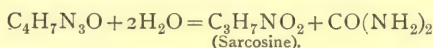
¹ The foregoing account is based on preliminary experiments. The writer hopes to publish fuller details later. (J. A. M.)

crystallisation, and from warm solutions in the anhydrous form. One part dissolves in about 11 parts of water at 16°, and in 102 parts of ethyl alcohol. Its solubility is much greater at higher temperatures. It is only very sparingly soluble in ether. It is a monacid base (dissociation constant at 40° = $3.69.10^{-11}$) forming very sparingly soluble picrates, phosphomolybdates, and phosphotungstates. It also forms sparingly soluble compounds with salts of the heavy metals, *e.g.* with $HgCl_2$, $Hg(NO_3)_2$, $AgNO_3$, and $ZnCl_2$. It is converted into creatine by the action of cold dilute alkalis, and conversely creatine is converted into creatinine with the loss of a molecule of water when heated with dilute mineral acids, *e.g.* N/I hydrochloric or sulphuric acids.

When heated with strong solutions of the alkalis, *e.g.* a saturated solution of barium hydrate, it is decomposed thus —



(C) Part of the creatinine is also hydrolysed thus :—



It is readily oxidised to oxalic acid and methyl guanidine by alkaline solutions or suspensions of mercuric oxide or cupric hydrate, and by potassium permanganate, yielding, amongst other products, oxalic acid and methyl guanidine $\left(NH:C \begin{smallmatrix} NH_2 \\ \nearrow \\ NHCH_3 \end{smallmatrix} \right)$. It therefore reduces Fehling's but not Benedict's or Nylander's solutions. In alkaline solution it reduces picric to picramic acid $[C_6H_2(NO_2)_2.NH_2.OH]$, and diaminonitrophenol $[C_6H_2NO_2.(NH_2)_2.OH]$. Upon this reaction Jaffe's test is based.

Preparation of creatinine from urine (Neubauer and Salkowski).

Render 240 c.c. of urine faintly alkaline with milk of lime, precipitate the phosphates by the addition of calcium chloride, avoiding excess, and filter after 15 minutes. Render the filtrate faintly acid with acetic acid, and concentrate to about 20 to 25 c.c. in a porcelain basin on the water bath. Extract the residue with 20 c.c. of ethyl alcohol, decant the alcoholic solution into a 100 c.c. flask, completely transfer the contents of the basin to the flask by washing with absolute alcohol, and finally make up to 100 c.c. Allow the alcoholic solution to stand for at least 12 hours, then filter, add about 1 c.c. of an alcoholic solution of zinc chloride (sp. gr. 1.20), a trace of sodium acetate, shake thoroughly, and allow to stand for 2 or 3 days. Filter off the zinc chloride compound, which separates as spherical masses of needle-shaped crystals, wash with cold 95 per cent. alcohol, then suspend the crystals in 50 c.c. of water, and set free the creatinine by boiling for 15 to 20 minutes with freshly precipitated lead hydrate,

again filter, concentrate the filtrate nearly to dryness, and extract the creatinine with warm absolute alcohol. Filter and concentrate the alcoholic solution until the creatinine crystallises out.

The creatinine thus obtained is not quite pure. Improved but more complicated methods have been devised by Folin and by Benedict.

The following tests may be applied to normal urine :—

1. WEYL'S REACTION.

Add a few drops of a freshly prepared dilute solution of sodium nitroprusside to a small quantity of urine. Then render alkaline with caustic soda. The solution acquires a red colour. Strongly acidify with acetic acid. The solution is practically decolorised. Heat the acid solution. It acquires a green tint and yields a blue deposit on standing. If ammonia be used instead of caustic soda, the colour of the solution remains unaltered (cf. acetone, p. 242).

Acetone gives a somewhat similar reaction (see p. 242). If necessary, urine may be freed from acetone by vigorous boiling.

2. JAFFE'S REACTION.

Add a small quantity of picric acid solution to some urine in a test-tube. Then render alkaline with caustic soda. The solution becomes deep red in colour. The colour change is aided by heat.

3. Fairly pure creatinine is required for this test.—Add a few drops of Fehling's solution to 1 or 2 c.c. of an aqueous solution of creatinine, and boil for several minutes. The solution is gradually decolorised; but no cuprous oxide separates out. The latter is probably kept in solution partly by unchanged creatinine and partly by the ammonia, which is simultaneously set free by the decomposition of the creatinine. Boil other portions of the solution of creatinine with Nylander's and Benedict's reagents.

The quantity of creatinine excreted in the twenty-four hours varies from .6 to 2 grms.

4. Indican.

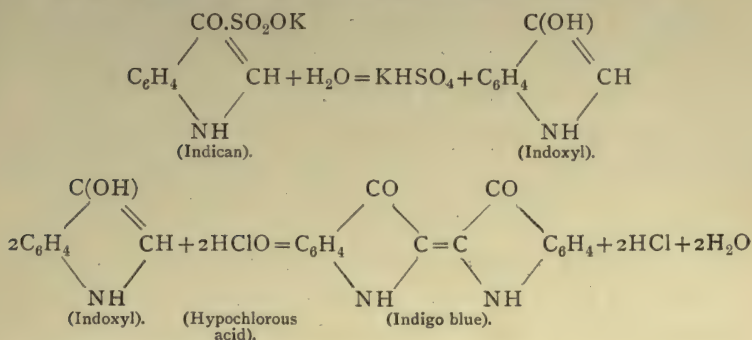
1. JAFFE'S TEST.

Acidify 10 c.c. of urine with an equal volume of concentrated hydrochloric acid. Then add 2 or 3 c.c. of

chloroform. Add, drop by drop, a dilute solution of calcium hypochlorite (1 in 20), shaking vigorously for some time after the addition of each drop. The chloroform acquires a bluish-violet colour, owing to the formation of indigo blue.

The following equations show the course of the reaction :—

In the presence of excess of hydrochloric acid—



The reaction is readily obtained with the urine of herbivora. In the case of normal human urine it is not invariably successful, even when carefully applied. The quantity of indican is increased in cases of partial or complete obstruction of the small intestine. It is also frequently much increased in cases of poisoning with oxalic acid.

2. Finely powdered commercial indigo blue may be used for the following tests :—

[(1) Heat a small quantity of indigo mixed with an equal amount of magnesium carbonate in a dry test-tube. The indigo sublimes, filling the tube with its purple-coloured vapour.]

The evolution of carbonic acid from the carbonate lessens the partial charring of the indigo, which would otherwise result.

[(2) Heat a small quantity of indigo with chloroform. It dissolves to form a blue solution.]

5. Hippuric acid or benzoylglycine ($\text{C}_6\text{H}_5\cdot\text{CO}\cdot\text{NH}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{OH}$).

For the following reactions, use hippuric acid prepared from horse's urine.

1. SOLUBILITIES.—It is only soluble with difficulty in cold, much more readily in boiling water.

Dissolve a few crystals in boiling water, and examine microscopically the crystals which separate out on cooling.

It crystallises in four-sided prisms, frequently arranged as rosettes. It dissolves readily in alcohol, with difficulty in ethyl ether, more readily in acetic ether.

2. The acid dissolves in alkalis.

To a small quantity of the acid, add dilute caustic soda or ammonia. Render the alkaline solution acid by the addition of hydrochloric acid. The hippuric acid is precipitated.

3. Heat a small quantity in a dry test-tube. The acid melts (at 187°C). On further heating, the melted mass becomes red, yields a white sublimate of benzoic acid, and gives off the odour of oil of bitter almonds.

The odour is due to the formation of phenylcyanide and hydrocyanic acid.

4. Add a few drops of concentrated nitric acid to a small quantity of hippuric acid, placed in a small test-tube of hard glass. Evaporate the mixture to dryness, and then heat the residue strongly. The characteristic odour of nitrobenzene develops.

The daily quantity of hippuric acid excreted in human urine varies much, mainly according to the diet (.1 to 1 gm.).

Preparation of hippuric acid from herbivorous urine (Roaf).

Dissolve 25 grms. of finely powdered ammonium sulphate in each 100 c.c. of cow's or horse's urine, acidify with 1.5 c.c. of concentrated sulphuric acid, allow the mixture to stand for 24 hours, and then filter off the crystals of hippuric acid, which have separated out.

6. Phenol ($\text{C}_6\text{H}_5\text{OH}$).

For the following reactions, use commercial phenol.

1. SOLUBILITIES.—The crystals form an oily fluid with

one-tenth their weight of water. They dissolve readily in water (1 in 15), and in alcohol and ether in all proportions. Phenol dissolves more readily in dilute caustic alkalies than in water, to form salt-like compounds which are insoluble in ether. These compounds may be decomposed by passing a current of carbon dioxide through their solutions. The chief colour reactions of phenol are given on page 132.

One additional reaction may be given.

Add a few drops of nitric acid to a dilute solution of phenol and heat. The solution becomes yellow owing to the formation of picric acid ($C_6H_2(NO_2)_3OH$). Cool and add ammonia. The yellow colour darkens to an orange (xanthoproteic reaction).

Phenol exists in the urine in ethereal combination with sulphuric acid and in combination with glucuronic acid. p-Cresol ($C_6H_4OH.CH_3$) is invariably present in the urine along with phenol. The quantity of both is increased in cases of increased bacterial decomposition of proteids within the intestines.

SEPARATION.—Several hundred c.c. of urine are rendered faintly alkaline with sodium carbonate, and evaporated down to small bulk. The concentrated urine is then rendered strongly acid with hydrochloric acid (about one-fifth of its volume), and distilled as long as phenol can be detected in the distillate by testing a small portion with Millon's reagent.

[7.] **Aromatic hydroxy acids** are present in normal urine. Although the amounts excreted are increased in cases of intestinal stasis, these acids are present even in the urine of animals in which the intestines are free from bacteria (Nuttall and Thierfelder), while the urine is completely free from phenol and cresol under the latter conditions.

PREPARATION (Baumann).

Acidify 250 c.c. of urine with acetic acid, and concentrate to about 25 c.c. on the water bath. Extract the residue at least thrice with ether, decant the ethereal solution into a separating funnel, and add gradually a dilute solution of sodium carbonate (waiting after each addition until effervescence of CO_2 has ceased) until the subjacent aqueous layer is distinctly alkaline to litmus paper. The hydroxy acids pass into the aqueous solution, while the greater part of the phenol and cresol is retained in the ether. The alkaline aqueous solution is acidified with sulphuric acid. Oily droplets of an acid substance, which gives the nitroso-indole colour reaction,

usually separate out at this stage (probably indole acetic acid). These, if present, are filtered off, and the hydroxy aromatic acids extracted from the filtrate by shaking up the latter with ether in a separating funnel. The ethereal solution is separated from the aqueous layer, and evaporated to dryness in a porcelain basin on the water bath. An aqueous solution of the residue will be found to give an intense red colour with Millon's reagent, a precipitate with bromine water, and an evanescent violet colour with ferric chloride.

The following hydroxy-aromatic acids have been isolated from urine—p-hydroxy-phenyl acetic, p-hydroxy-phenyl propionic, and p-hydroxy-phenyl lactic acids ($[\alpha]_D = -18^\circ$). All these acids are products of the decomposition of tyrosine. The last-mentioned acid has only been isolated from pathological urines, and is the only optically active member of the group.

Homogentisic acid (1-4-dioxyphenyl-3-acetic acid), which occurs in the urine in the rare inborn error of metabolism known as alcaptonuria, may be isolated in a similar manner with the substitution of a mineral acid, e.g. HCl for acetic.

[8. Pigments of the Urine.]

1. UROCHROME is the normal pigment of the urine.

GARROD'S METHOD OF SEPARATION depends on the fact that urochrome can be extracted from the urine after saturation with ammonium sulphate.

Saturate some urine with ammonium sulphate. Filter from the precipitate which forms, and extract the filtrate with one-fifth its volume of absolute alcohol. Part of the ammonium sulphate is precipitated, and a clear yellow layer of alcohol, containing the pigment, forms above the saturated salt solution. The pigment may be purified by solution in water and repetition of the process of saturation. The pigment shows no absorption bands, and does not show a green fluorescence in presence of ammoniacal zinc chloride.

2. UROBILIN.¹—Fresh normal urine contains little, if any,

¹ For class purposes urine may be used to which urobilin prepared from fæces has been added. In order to prepare the pigment, extract the fæces with water, then acidify the filtrate with dilute sulphuric acid and saturate with ammonium sulphate. Filter off the precipitate and dissolve it in acid alcohol.

urobilin. A chromogen of urobilin is present in small amount. The quantity of urobilin is much increased in certain pathological conditions.

METHOD OF SEPARATION (GARROD).—Precipitate the uric acid by saturating the urine with ammonium chloride. Filter. Acidify the filtrate with dilute sulphuric acid, and saturate with ammonium sulphate. Then shake up the saturated salt solution in a separating funnel with an equal volume of a mixture of 1 volume chloroform to 2 volumes of ether. Separate the solution of urobilin in ether and chloroform, and shake up with water rendered faintly alkaline with ammonia. The pigment passes into watery solution. Separate its aqueous solution from the ether and chloroform, and perform the following tests:—

(1) (a) *Render part of the aqueous solution alkaline with ammonia, and add a few drops of zinc chloride solution. Note the distinct green fluorescence. Examine the solution spectroscopically. Note an absorption band in the green-blue between b and F.*

(b) *Acidify the aqueous solution of the pigment with dilute hydrochloric acid, and shake up with amyl alcohol. The alcohol extracts the pigment. Examine the alcoholic solution. Note a broad absorption band in the green-blue between b and F, and extending to the violet side of F. Compare with the spectroscopic characters of (a).*

(2) Nencki and Rotschy's test. The following method is a simpler one, but yields an impure solution of urobilin:—

Add 2 or 3 drops of hydrochloric acid to 10 c.c. of urine, then mix gently with 6 c.c. of amyl alcohol (too vigorous shaking produces a somewhat persistent emulsion). Pipette off the alcoholic layer and examine with the spectroscope. Then add a few drops of an ammoniacal solution of zinc hydrate in ethyl alcohol (1 grm. zinc chloride and 100 c.c. strongly ammoniacal alcohol). If urobilin be present, the alcoholic solution will show well-marked green fluorescence, and the characteristic spectrum of urobilin in alkaline solution.

If the emulsion prove troublesome, the mixture of amyl alcohol and urine may be filtered through paper previously moistened with amyl alcohol (Huppert).

3. HÆMATOPORPHYRIN and its chromogen are present in small amount in normal urine. For its detection, at least 500 c.c. urine should be used. Add 200 c.c. of 10 per cent. caustic soda to 1 litre urine. The precipitate of earthy phosphates which contains the hæmatoporphyrin is allowed to settle. The supernatant fluid is then siphoned off, and the precipitate transferred to a filter paper, washed with water, and extracted with alcohol, acidified with hydrochloric acid. The alcoholic solution of the pigment is then filtered and examined spectroscopically. It shows the spectrum of acid hæmatoporphyrin.

4. PATHOLOGICAL PIGMENTS.

(1) Melanin and its chromogen (melanogen) sometimes occur in the urine of patients with melanotic growths. The urine may be dark-coloured when fresh, or only after exposure to the air, or on the addition of oxidising agents, such as bromine water.¹

(2) When phenol has been absorbed from surgical dressings, it is excreted largely as polyhydric derivatives of benzene, such as pyrocatechol (1-2-dihydroxybenzene) and hydroquinone (1-4-dihydroxybenzene). These are united with sulphuric acid as ethereal sulphates. When urine containing these bodies is rendered alkaline, it absorbs oxygen, and becomes successively green, greenish-brown, brown, or even black.

(3) Urine containing homogentisic acid undergoes similar colour changes when treated with alkali.²

(4) The urine may also contain foreign pigments derived from drugs or food-stuffs. Some of these will be considered later.

[9. The enzymes of Normal Urine.]

(1) Detection of Pepsin.—*Suspend some well-washed fibrin in water, immerse the vessel in boiling water for at least 15 minutes to destroy adherent pepsin, cool, and remove the fibrin. Place a small piece of this fibrin in 30 c.c. of urine placed in a small flask (labelled A), and another piece in 30 c.c. of the same urine, which has been previously boiled and cooled, placed in a flask (control B). Add a few drops of toluene to each sample, and leave both in the ice-chest for 24 hours. Remove the fibrin flocculi from (A) and (B) and wash each thoroughly with water to remove salts and other urinary constituents. The pepsin absorbed by fibrin (A) is not removed by washing with water. Then immerse the flocculi of fibrin (A and B) in 20 c.c. portions of 0.2 per cent. hydrochloric,*

¹ Neubauer-Huppert's "Analyse des Harns," 11th Ed., 1910, pp. 1313-1317.

² See Huppert, *loc. cit.*, pp. 793, 796, and 850.

add a little toluene to each, and leave in the incubator at 37° to 40° for 12 hours. The fibrin (A) will be found to have dissolved more or less completely, while (B) has swollen up but not dissolved. The filtrates from (A) and (B) are then examined as described in the chapter on Gastric Digestion.

(2) Detection of Trypsin.—Use 0.2 per cent. sodium carbonate instead of 0.2 per cent. hydrochloric acid; but in other respects follow the procedure already described in (1).

Trypsin is not usually present in normal urine.

(3) Detection of Amylase.—Add 1 c.c. of freshly prepared 2 per cent. starch mucilage, and a little toluene to 20 c.c. of urine placed in a small flask (A). Carry out the same procedure with a sample of previously boiled and cooled urine placed in a flask (B) to serve as a control. Place both flasks in the incubator at 37° to 40° for 6 hours. Then test portions of each with iodine and with Fehling's solution (see page 102 for methods of examination).

Normal urine always contains amylase. The amount excreted is increased after meals, and is also influenced by various pathological conditions.

Although the laws governing the chemical composition of the urine are of the utmost practical importance, limits of space have rendered adequate reference to them impossible. Students are advised to read the chapters on Metabolism in a text-book of Physiology, and, if time permits, such works as E. P. Cathcart's "Physiology of Protein Metabolism" (Longmans, Green & Co.) concurrently with their practical work.

CHAPTER XV

PATHOLOGICAL CONSTITUENTS OF URINE AND URINARY SEDIMENTS

I. PROTEIN

I. Albumin.

(a) HEAT COAGULATION.

To a small quantity of filtered urine add at least one-sixth its bulk of a saturated solution of sodium chloride, and heat to boiling point. The precipitate which forms may consist of albumin, or earthy phosphates, or both. To each 10 c.c. of the hot urine add two drops of 33 per cent. acetic acid. The precipitate of earthy phosphates, if present, dissolves; while the albumin remains undissolved in the form of coagula.

The following modification of the foregoing method is easily and rapidly carried out, and appears to be more delicate and free from fallacies than any of the previous methods of detecting coagulable protein (Bang).¹ This method is based on the principle that the heat coagulation of serum albumin and serum globulin is most nearly complete at certain hydron concentrations (p_H for maximal heat coagulation of serum albumin, 5.7, and for serum globulin, 5.85). The reaction of the urine is converted to that best adapted for the coagulation of both proteins by the addition of a solution containing acetic acid and sodium acetate.

Add 1 c.c. of the reagent² to 10 c.c. of the urine placed in a test-tube, heat to the boiling point, and maintain at about the boiling point for about half a minute. If the urine contains more than traces of coagulable protein, a flocculent precipitate will separate out. If only traces of protein be present (.005 to .01 grm. per 100 c.c. urine) the

¹ "Lehrbuch der Harnanalyse," 1918. Publisher, J. F. Bergmann.

² Dissolve 118 grms. of sodium acetate and 56.5 c.c. of glacial acetic acid in water, and dilute to a litre. The reagent remains unchanged for an indefinite time.

urine will become merely opalescent, but if allowed to stand for a few minutes, a finely flocculent precipitate will gradually separate. If the test be negative, the urine either contains no coagulable protein or less than .005 grm. per 100 c.c.

(b) HELLER'S TEST.

Place a little concentrated nitric acid in a test-tube, and pour the urine slowly down the side of the inclined test-tube. The urine will form a layer above the nitric acid. If albumin be present, a whitish precipitate or turbidity will form at the plane of junction of the two fluids. Note the red or reddish-violet ring at the plane of junction, due to presence of indigo-red and indigo-blue. If the urine be concentrated, a crystalline precipitate of urea may form at the plane of junction. The obvious crystalline nature of the precipitate renders it readily distinguishable from an albuminous precipitate. In a urine rich in urates, a precipitate of uric acid may also form. It usually also forms at the plane of junction of the nitric acid and urine. Its appearance may be prevented by diluting the urine before performing Heller's test.

(c) ROBERTS' TEST.—Place about 2 c.c. of the reagent (prepared by mixing 1 vol. of concentrated nitric acid with 5 vols. of a saturated solution of magnesium sulphate) in a test-tube, and allow one or two c.c. of urine to flow gently from a pipette on to the surface of the reagent. A white ring at the junction of the fluids indicates the presence of albumin. No coloured rings are formed, such as may occur in test (b).

(d) ACETIC ACID and POTASSIUM FERROCYANIDE (see p. 74).

(e) The colour reactions for proteins cannot be applied to the urine directly.

Filter off the coagulum produced by heat, suspend one portion of it (a) in a small quantity of Millon's reagent, and heat. The coagulum acquires a red colour. Dissolve, with the aid of heat, the other portion (b) of the coagulum in 5 per cent. caustic soda, and add a drop or two of very dilute solution of cupric sulphate. The solution acquires a violet colour (biuret).

(f) *A large number of other protein precipitants have been used. One of the most useful of these is salicyl sulphonic acid. Add a few drops of a 20 per cent. solution of the acid to a few c.c. of filtered urine. A precipitate or cloud indicates the presence of albumin.*

2. Detection of albumin and globulin.

Neutralise some urine, filter, and add an equal volume of a saturated solution of ammonium sulphate. Filter off the precipitate of globulin which forms. Dissolve it in 2 per cent. sodium chloride, heat, and acidify with dilute acetic acid. A coagulum forms. Heat also the filtrate from the precipitate of globulin produced by half saturation with ammonium sulphate, and acidify with dilute acetic acid. A coagulum of albumin separates out.

[3. Albumose in presence of coagulable protein.]

Add 8 parts finely powdered ammonium sulphate to 10 parts urine. Heat to the boiling point. The coagulable protein is rendered permanently insoluble in water and neutral salt solutions. Filter while hot. Extract the precipitate with alcohol to free it from urobilin, which, like albumoses, gives the biuret reaction. Then extract the precipitate with boiling water, which dissolves the albumoses, leaving the coagulated protein undissolved. Test the filtrate by biuret and xanthoproteic reactions.

[4. Bence-Jones' Protein.]

This protein is found in the urine in certain cases of multiple myelomas ("myelopathic albumosuria").

Gradually heat the urine (faintly acidified if necessary with dilute acetic acid) in a test-tube immersed in a beaker of water. The urine becomes turbid at about 45°. The precipitate usually increases until the temperature of 60° is reached, and then gradually dissolves as the temperature is raised to the boiling point. Cool the tube under the tap. The precipitate reappears.

5. Peptone.

True peptone is rarely if ever found in the urine.

6. Nucleo-albumin and so-called **mucin** of the urine. Normal urine contains only traces of mucinoid substance. If a specimen becomes opalescent, or turbid, or yields a precipitate on acidification with acetic acid in the cold, it should be examined to find out the nature of the protein present. The salts of the urine hinder the precipitation with acetic acid, and should therefore be first removed by dialysis. Larger text-books should be consulted for details with regard to the nature and methods of separation of the mucinoid substances which may be present in the urine.

[7. Chondroitin Sulphuric Acid (Mörner),

which is a non-dialysable constituent of normal urine, may be mentioned here. It is not a protein; but forms compounds with proteins, and is also a reversible colloid (*see* Neubauer-Huppert's "Analyse des Harns," pp. 1256 to 1264).]

[8. Pus in Urine.]

The leucocytes may be separated by centrifugation and examined by histological methods. If the urine be alkaline, the leucocytes undergo more or less complete disintegration, and histological examination is rendered difficult. In such cases the following test is useful :—

Add a small quantity of solid caustic alkali or a drop or two of a concentrated solution of NaOH or KOH to the urinary sediment. If pus be present in the sediment a jelly-like mass is obtained. Dilute the jelly with a little water, and acidify with dilute acetic acid. A flocculent precipitate of nucleoprotein separates. Urine containing pus always contains coagulable protein.

9. Removal of Protein from the Urine.

When a urine contains protein, removal of the latter is frequently essential as a preliminary to the further examination of the urine (*e.g.* with the polarimeter).

In many cases (*e.g.* before testing for sugars) albumin or globulin may be removed by heat coagulation. The chief objection to this method is that certain substances, which sometimes occur in urine (*e.g.* aceto-acetic acid), are decomposed, or volatilised by heat. Another objection to the method is that the presence of acetic acid may interfere with certain colour reactions, *e.g.* the ferric chloride test for aceto-acetic acid.

A colloidal solution of ferric hydrate, which precipitates proteins completely, may also be employed. All the colloids of the urine are precipitated by this reagent. The latter fact excludes its use in certain

cases. Another objection to this reagent is that it usually contains some ammonia.

After shaking an albuminous urine with kaolin powder (1 part to 3 parts of urine), and filtering, the filtrate will be found to be free from albumin. Hæmoglobin is not precipitated by this method.

Protein may be removed by mixing a sample of the urine with animal charcoal (about 1.5 gr. for 20 c.c of urine) and 10 per cent. of ethyl alcohol, shaking thoroughly and then filtering. The filtrate is usually free from protein and pigments. The addition of alcohol prevents adsorption by the charcoal of all other urinary constituents except protein and pigments. The precipitation of protein by this method is liable to be incomplete when a large quantity of protein is present.

II. BLOOD.

Note the general appearance of the urine. The urine appears more or less turbid, and has a reddish, yellowish-red, red-brown, or even dark brown tint. Much depends on whether the hæmorrhage has been recent or remote. The colouring matter of the blood may be retained within the red blood corpuscles (hæmaturia), or dissolved in the urine either in the form of unchanged hæmoglobin (hæmoglobinuria), methæmoglobin (methæmoglobinuria), or hæmatin.

(a) *Separate any formed constituents, which may be present, by means of the centrifuge, and examine the deposit microscopically for more or less altered red blood corpuscles and remains of their stromata.*

(b) *Do Guaiac reaction, see p. 160.*

N.B.—The urine gives a somewhat similar reaction after the administration of iodides.

(c) SPECTROSCOPIC EXAMINATION for oxyhæmoglobin or methæmoglobin.

(d) HELLER'S TEST.

Render some urine alkaline with caustic soda and heat. A precipitate of earthy phosphates forms, coloured red owing to adhering hæmatin.

Sources of Error.

A number of purgatives, *e.g.* cascara sagrada, senna, aloes, and rhubarb, contain anthraquinone derivatives, which become red on the addition of alkali, and by adsorption colour the phosphate precipitate red.

(*e*) A very delicate test consists in the preparation of *hæmochromogen*, from the blood pigment present.

To some urine add one-fourth its bulk of concentrated caustic soda. Heat to boiling. Cool, filter, and add ammonium sulphide to the filtrate. On spectroscopic examination, the solution will show the characteristic absorption bands of hæmochromogen.

[(*f*) STANNOUS TEST (J. A. Milroy).]—*Add 2 to 3 c.c. of butyric acid to 10 c.c. of the urine containing blood, mix thoroughly, and boil. Then saturate the solution with sodium chloride. The butyric acid is "salted out," and rises to the surface as an oily layer, which may be coloured brown by (oxy)hæmatin, if a considerable quantity of blood be present. Transfer the butyric acid with a test pipette to a dry test-tube, add 3 to 4 drops of 2 M. stannous chloride (or a small quantity of solid stannous chloride, a small granule of metallic tin, and one drop of concentrated hydrochloric acid), boil for about 2 minutes, then add a little solid sodium acetate, and again boil. On spectroscopic examination the two well-defined absorption bands of the stannous compound will be clearly seen. Filtration is usually unnecessary, especially if a little time be given for the solution to clear; but, if the butyric acid solution of the pigment be so turbid that examination with the spectroscope is difficult, the solution may be filtered through a small filter paper.*

One part of blood in 10,000 parts of urine can be detected by this test, which can easily be carried out in about five minutes. The delicacy of the test can be still further increased by certain modifications, but only at the expense of simplicity. It is a much more delicate test for blood than (*e*), and equally specific.

(*g*) BENZIDINE TEST (Adler), see p. 160.—When applied to urine, this test is much less delicate than with dilute solutions of blood pigment in water. Urine containing 1 part of blood in 1000 was found not to give a distinctly positive result.

III. BILE.

See pp. 146-148 for tests for bile pigment and bile salts. Perform also the surface tension test with flowers of sulphur.

A. BILE PIGMENTS.

The following tests may be employed in addition to Gmelin's test :—

1. ROSENBACH'S MODIFICATION OF GMELIN'S TEST.

Filter the urine containing bile, spread out the filter paper, and place a drop of nitric acid containing nitrous acid on it.

The following coloured zones appear round the drop from within outwards—yellow-red, violet, blue, and green.

2. *Incline a test-tube containing a little icteric urine, and let some tincture of iodine flow slowly down the side of the tube. A green ring appears at the plane of junction of the two fluids.*

3. HUPPERT'S REACTION.

[Add to the bilious urine a solution of barium hydrate, or milk of lime, so long as a pigmented precipitate continues to form. Filter and extract the precipitate on the filter paper with hot alcohol acidified with sulphuric acid.]

A clear green solution is obtained. This method is successful in urines which yield a doubtful reaction with Gmelin's test.

[4. COLE'S TEST.]—"Add two drops of saturated magnesium sulphate to 10 or 15 c.c. of urine, boil, then add 10 per cent. barium chloride solution, drop by drop, boiling between each addition. Continue to add the barium chloride until no further precipitate is obtained. Allow the tube to stand for a minute. Pour off the supernatant fluid as cleanly as possible or use a centrifuge. To the precipitate add 3 to 5 c.c. of 97 per cent. alcohol, 2 drops of concentrated sulphuric acid, and 2 drops of a 5 per cent. aqueous solution of potassium chlorate. Boil for half a minute and allow the barium sulphate to settle. The presence of bile pigments is indicated by the alcoholic solution being coloured greenish-blue. To render the test more delicate, pour off the alcoholic solution from the barium sulphate into a dry tube. Add about one-third its volume of chloroform, and mix. To the solution add about an equal volume of water, place the thumb on the tube, invert once or twice, and allow the chloroform to separate. It contains the bluish pigment in solution."

B. BILE SALTS.

Bang and Laurin's test is based on the fact that the bile acids are completely precipitated by saturation with magnesium sulphate in acid solution. When the quantity of bile salts is small, a protein solution may be added with advantage. The protein precipitate carries the bile acids with it.

Add 2 or 3 drops of blood serum to 20 or 50 c.c. of urine, saturate with magnesium sulphate, add 1 to 2 drops of concentrated hydrochloric acid, boil the mixture and filter. If the urine be highly coloured, wash the precipitate with saturated magnesium sulphate, then partially dry it by pressing between filter papers, extract the precipitate with 10 to 15 c.c. of boiling alcohol in a test-tube, decant the alcoholic solution into another test-tube, add a little solid barium hydrate, boil, filter, and concentrate the filtrate to dryness by heating on the water bath. Add 1 drop of 1 per cent. cane sugar solution, and 2 to 3 c.c. of concentrated hydrochloric acid to the residue, mix thoroughly and heat gradually. A violet colour reaction indicates the presence of bile acids. An alternative method is to test the residue by the Pettenkofer reaction.

IV. UROERYTHRIN.

This pigment gives the red colour to deposits of urates.

DETECTION.

[Collect the sediment on a filter. Dissolve in water with the aid of gentle heat, and extract with amyl alcohol.]

This method yields an impure product, since the amyl alcohol also dissolves urobilin and hæmatoporphyrin. Examine the amyl alcohol solution spectroscopically. Two bands are present—one close to the red side of E, and one between *b* and F. The best method of separation is that due to Garrod, for which larger text-books may be consulted.

V. CARBOHYDRATES AND REDUCING SUBSTANCES.

1. **Glucose** is present in normal urine, but only in minute traces (about 0.05 per cent.). The continuous excretion of considerable quantities of glucose is characteristic of diabetes mellitus. The urine in typical cases of diabetes mellitus is

large in volume (3 to 10 litres), pale in colour, of high specific gravity (1030 to 1040 or higher), and contains sugar. The percentage amount of the physiological constituents of the urine is less than normal, while their daily quantity is increased.

TESTS—

(a) TROMMER'S and FEHLING'S TESTS (see pp. 13 and 14).

When the quantity of glucose present is large, there is no difficulty in obtaining a definite positive reaction; but if the quantity of glucose be small, its detection is difficult, since normal urine contains substances, *e.g.* uric acid and creatinine, which reduce Trommer's reagent. Another source of fallacy is, that after the administration of certain drugs, *e.g.* antipyrine, chloral, salol, and others, compounds of glycuronic acid occur in the urine. These also reduce Trommer's reagent.

(b) BISMUTH TEST.

To 10 c.c. urine add 1 c.c. of Nylander's reagent, and heat for some minutes. If the quantity of sugar present be large, the solution becomes yellow, then brown, then darker brown, and finally a black precipitate of metallic bismuth separates out. If the quantity of sugar be small, the urine merely darkens, and, after standing for some time, a black deposit of metallic bismuth separates out. The test will indicate .05 per cent. glucose.

Nylander's reagent has the advantage of not being reduced by creatinine or uric acid. It is reduced by glycuronic acid.

If much albumin be present, a fallacy arises, owing to the formation of sulphide of bismuth from the loosely combined sulphur of the protein. The urine should therefore be freed from protein by heat coagulation before performing the test.

(c) COLE'S TEST FOR SMALL AMOUNTS OF GLUCOSE IN URINE.—“In a large test-tube place about 1 gm. absorbent charcoal. Add 10 c.c.

of urine, shake, heat to boiling and then cool under the tap. Shake at intervals for 5 minutes. Filter through a small paper into a dry test-tube. To the filtrate add 4 drops of pure glycerol and 0.5 gm. of anhydrous-sodium carbonate. Shake and heat to boiling. Maintain the boiling for exactly 50 seconds. Immediately add 4 drops of a 5 per cent. solution of crystalline copper sulphate, shake to mix, and allow the tube to stand without further heating for one minute. With normal urine the fluid remains blue. If glucose is present to the extent of 0.03 per cent. above the normal amount in urine the blue colour is discharged and a yellowish precipitate of cuprous hydroxide forms."

NOTES.—1. Treatment with adsorbent charcoal removes practically the whole of the urates, creatinine, and pigments that interfere with Fehling's test. It also adsorbs so much of the normal amount of glucose present that the filtrate from normal urine fails to give a reduction.

2. Should the specific gravity of the urine exceed 1.025 it is advisable to use 5 c.c. of urine + 5 c.c. of water.

3. The test is not given by chloroform or glycuronates, it is given by pentoses.

(d) BENEDICT'S TEST.—Add 5 to 10 drops of the urine to 5 c.c. of Benedict's (qualitative) reagent, boil vigorously for 2 or 3 minutes, then allow to cool. If glucose be present, a red, yellow, or green precipitate separates, the colour of the precipitate being dependent on the amount of glucose.

Benedict's reagent is not readily reduced by creatinine or uric acid.

(e) FERMENTATION TEST with ordinary yeast (see p. 18).

If the urine be alkaline, it should first be faintly acidified with tartaric acid. This test succeeds readily when the quantity of sugar is large; but if the quantity of sugar indicated by the bismuth test be very small, the fermentation test may yield a negative result. In this case, after the urine has been allowed to ferment with yeast for twenty-four to forty-eight hours, it is again tested with Nylander's reagent. If the result of testing with Nylander's reagent after fermentation be negative, it may be concluded that glucose was originally present. If, on the other hand, on testing the urine (after fermentation) with Nylander's reagent, a positive result is obtained, probably the reduction is due to other reducing substances, *e.g.* glycuronic acid or lactose.

(f) PHENYLHYDRAZINE TEST.

Before applying the test, it is best, if possible, to estimate the quantity of glucose present, and then use for the test

the quantity of phenylhydrazine required by the equation expressing the reaction. If not, the test may be performed in the following way (Neumann):—

Take 5 c.c. of urine in a special test-tube, with bulb on it, and marks for 3, 5, and 7 c.c. in the lower part of the tube. Add 2 c.c. 50 per cent. acetic acid that has been saturated with sodium acetate, and two drops of pure phenylhydrazine. Evaporate down to 3 c.c., rapidly cool, and then again warm. It is then left to cool slowly. Crystals separate out in a very short time, even when percentage is small.

(g) POLARISATION TEST is of great value for distinguishing glucose from the compounds of glycuronic acid or from lævulose, since both of these are lævo-rotatory.

(See pp. 32-33 for other tests.)

2. **Fructose** is sometimes present in urine, either alone or much more frequently along with glucose. If the urine be alkaline, glucose may be partly converted into fructose (Lobry de Bruyn and van Ekenstein).

The following are the chief properties of urine containing fructose. The urine gives the reduction tests, is fermentable with yeast, and either lævorotatory, or when dextrorotatory, yields higher titration values than those obtained by polarimetric examination. The inference that fructose is present cannot be justifiably drawn from these facts, unless the presence of β -hydroxybutyric and of paired compounds of glycuronic acid can be excluded.

After fermentation with yeast the urine should not show a greater lævorotation than normal urine, if β -oxybutyric acid be absent, and if the paired compounds of glycuronic acid do not exceed the normal amount.

The following tests are given only by fructose and by carbohydrates (*e.g.* cane sugar or inulin), which yield fructose. The only source of error depends on the fact that, on prolonged heating with acid, glucose is partly converted into fructose, and the latter is decomposed by the further action of the acid yielding a substance (ω -hydroxymethylfurfural) which gives a red colour with resorcinol:—

(1) ROSIN'S MODIFICATION OF SELIWANOFF'S TEST.

Mix 2 c.c. of urine with 2 c.c. of concentrated hydrochloric acid, add a few granules of resorcinol, and heat to the boiling point. If the characteristic red colour be obtained, cool the solution, and add gradually a concentrated solution of sodium carbonate until effervescence ceases. During the later stages of neutralisation solid sodium

carbonate may be added instead of the solution. Then shake up the solution with 2 or 3 c.c. of amyl-alcohol. An orange-red alcoholic solution showing faint green fluorescence is obtained if fructose be present. The colour becomes rose-red on the addition of a few drops of ethyl-alcohol. Examine the alcoholic solution with the spectroscope, and note an absorption band in the green extending from E to b.

(2) BANG'S TEST.—Add a granule of bile salts to 1 or 2 drops of the urine, then add about 3 c.c. of concentrated hydrochloric acid, mix thoroughly, and boil for one-half to one minute. If fructose be present, a well-marked violet colour develops which deepens on standing. 0.02 mg. of fructose can be detected by this method.

This reaction is not given by glucose, galactose, maltose, lactose, or the pentoses. Glucose only yields a faint violet colour after prolonged boiling. The pigments of the urine do not usually interfere with the reaction, since only a small quantity of urine is required for the test.

3. d-Galactose.—This sugar is occasionally found usually with lactose in the urine of infants, especially those suffering from gastro-intestinal disorders. The tolerance for galactose is also reduced in certain pathological conditions of the liver. When administered to a diabetic patient it is converted into glucose.

The reactions given on page 22, 3, may be used for its detection. The osazone may be separated from urine by the method described for glucose. Galactosazone (m.p. 196° to 197°) separates as yellow needles, which are thicker than the similar compound of glucose (m.p. 205° to 210°). They are even more easily distinguished from the very fine needles of lactosazone (m.p. 200°).

4. d-, l-, and i-Mannose.—The alimentary tolerance limit for these sugars appears to be a low one in rabbits, a large proportion of the sugars passing into the urine (Neuberg and Mayer). The assimilation limit for man does not appear to have been determined.

5. Lactose is occasionally present in the urine of women during pregnancy and the nursing period. Alimentary lactosuria may occur when "the assimilation limit" for a given individual is exceeded. The tolerance for lactose is distinctly reduced in certain pathological conditions, especially in certain intestinal disorders affecting infants. (See pp. 26-28 for the points of difference between it and glucose.)

Chief characteristics of urine containing lactose. The urine gives the reduction tests with Fehling's, Nylander's, and Benedict's reagents, but does not ferment with yeast unless a fermentable sugar is also present. It is dextrorotatory. Lactose is hydrolysed to glucose and galactose by emulsin. The consequent alteration in rotation, and the partial fermentability with yeast after the action of emulsin might be utilised in its

detection. It may be isolated from urine by precipitation with lead acetate and ammonia (for details see Hofmeister, "Zeitschr. f. physiol. Chem.," vol. i. p. 105), and identified by the reactions already described (pp. 26 to 28).

COLE'S TEST.—Add 25 c.c. of the urine to 1 gm. of good charcoal, mix, boil for a few seconds, cool, and shake at intervals for 10 minutes. Filter through a small paper, preferably with the aid of the filter pump, and extract the deposit of charcoal with 10 c.c. of water and 1 c.c. of glacial acetic acid. Boil the charcoal with the dilute acid for about 10 seconds, and filter the hot solution into a test-tube containing phenylhydrazine hydrochloride (as much as will lie on a shilling), and twice as much sodium acetate. Mix thoroughly, filter from any insoluble oily residue, and immerse in a boiling water bath for 45 minutes. Remove the tube, and allow it to stand at room temperature for at least one hour (preferably longer). Pipette off a little of the deposit, if any, and examine under the high power of a microscope for crystals of lactosazone.¹

6. Sucrose (or cane sugar) is excreted in the urine when injected subcutaneously; but is rarely if ever found in the urine when taken as a food. It can be easily detected by polarimetric examination, the resorcinol test, and by reduction tests applied before and after inversion with dilute acid or invertase.

7. Pentoses ($C_5H_{10}O_5$) are incompletely assimilated, 10 to 50 per cent. of the quantity taken being excreted in the urine. "Alimentary pentosuria," therefore, occurs occasionally, when certain fruits (*e.g.* plums, apples, and cherries), which contain pentoses, have been taken. Pentoses are also excreted along with glucose in certain cases of diabetes even when pentoses have been excluded from the diet. The pancreas is probably the source of this pentose. Finally, chronic pentosuria occurs as a rare inborn error of metabolism. In this condition large quantities of pentoses up to 36 grms. per day are excreted even when substances yielding pentoses are excluded from the diet. The source of the pentose in this disorder is uncertain. The total quantity of pentose in the tissues of the body is only about 10 grms. The large quantity of pentose frequently excreted in this disease cannot, therefore, be directly derived from the pentose of the tissues.

CHARACTERS OF URINE CONTAINING PENTOSEs.

When boiled with Fehling's (or Benedict's) solution reduction does not occur immediately, but only after prolonged boiling and then suddenly. According to Neuberg, this phenomenon is due to the fact that the pentose is present in the urine as a ureide [$NH_2.CO.N:CH.(CHOH)_3.CH_2OH$], and reduction does not occur until the ureide has been hydrolysed. The urine does not undergo fermentation with yeast.

¹ See Cole's "Physiological Chemistry," p. 314.

In chronic pentosuria the urine is usually found to be either optically inactive, or slightly dextrorotatory. In alimentary pentosuria the urine is optically active, the amount and direction of rotation varying with the form of pentose present. The urine also gives the colour reactions described on pages 23 and 24. Like all carbohydrates, the pentoses give the Molisch reaction. The pentoses can be easily distinguished from the hexoses by means of the foregoing reactions, but many of the reactions just mentioned are also given by glycuronic acid and its compounds. The following modification of the orcinol reaction is more delicate and specific than the original test.

BIAL'S REACTION FOR PENTOSSES.—*Heat 4 to 5 c.c. of the reagent (1 grm. of orcinol dissolved in 500 c.c. of hydrochloric acid of sp. gr. 1.15 plus 25 drops of 10 per cent. ferric chloride solution) to the boiling point. Withdraw the test-tube from the flame, add at most 1 c.c. of urine, mix thoroughly, and allow to stand for a few minutes. The development of a green colour indicates the presence of pentoses.*

8. Glycuronic acid, which is a product of the incomplete oxidation of glucose, does not occur in the urine as such, but always in combination as a glucoside. 100 c.c. of normal urine contain about 0.004 grms. of glycuronic acid (Neuberg and Mayer). The glycuronic acid of normal urine is present as conjugated compounds with phenol, cresol, and other phenols, indoxyl, scatoxyl, and possibly sometimes with benzoic acid. The quantity is therefore increased in cases of intestinal stasis. Paired compounds of glycuronic acid are also excreted after the administration of a large number of organic substances, *e.g.* aliphatic, and especially aromatic alcohols, also aldehydes and aromatic acids. The aldehydes, *e.g.* chloral hydrate, are reduced to alcohols before combining with the glycuronic acid. The most important compounds of glycuronic acid are those with chloral, camphor, menthol, thymol, benzoic acid, many volatile oils, *e.g.* those of eucalyptus and copaiba, a number of aromatic hydrocarbons (which are oxidised in the body to alcohols, and then combined with glycuronic acid), and a large number of drugs, *e.g.* salicylates, senna, rhubarb, aloes, sulphonal, tetronal, antipyrine, quinine (in large doses), and morphine (large dose).

For the practical study of conjugated glycuronates, urochloralic acid may serve as a type. A moderate dose of chloral hydrate (about 1 grm.) was taken at night, and the morning's urine was collected and examined in the following way:—

(1) *Make up 90 c.c. of urine to 100 c.c. by the addition of ethyl-alcohol, add 4 grms. of finely powdered animal charcoal, shake, allow to stand for about an hour, and then filter. Part of the colourless filtrate is then transferred to a 2 decimeter tube, and examined with the polarimeter. A slight lævorotation (not more than -0.05° in the case examined) will be detected. Nearly all the conjugated glycuronates are lævorotatory. The $[\alpha]_D$ of the compound with trichlorethyl alcohol (urochloralic acid) has the value*

—69.6°. The quantity of this substance in the urine examined must therefore have been small. According to Borntraeger, α_D after therapeutic doses of chloral is about —1°. Free glycuronic acid is dextro-rotatory ($[\alpha]_D = +19.4^\circ$ for concentrations of 3 to 14 per cent.). The urine should therefore be dextrorotatory after hydrolysis with dilute mineral acid or with emulsin.

(2) Apply the chief reduction tests to samples of the urine. Reduction of Fehling's, Benedict's, and Cole's solutions occurred. The result with Nylander's reagent was doubtful. A marked delay in reduction was noted. The immediate reduction on heating was relatively slight, but on standing marked reduction developed. Probably the urine behaved thus, because the conjugated glycuronate has less reducing power than the glycuronic acid formed by its hydrolysis. Many of the conjugated glycuronates have no reducing power until hydrolysed.

(3) Apply the orcinol and phloroglucinol reactions. The former test did not give a typical result, the solution of pigment in amyl-alcohol being red with a faint greenish tint; but on spectroscopic examination the same absorption band was found as that which pentoses yield. The phloroglucinol reaction gave a red coloured alcoholic solution showing very faintly the absorption band, which is also given by pentose under the same conditions.

(4) Add about one volume of concentrated sulphuric acid to three volumes of urine, boil vigorously, and hold a paper impregnated with aniline acetate in the vapours given off. The paper becomes red (furfural reaction). The latter colour reaction is given by the pentoses with greater intensity than with glycuronates, since pentoses yield a higher percentage of furfural than glycuronates. The colour reaction with aldohexoses is very faint, but distinct with ketohexoses.

(5) TOLLEN'S TEST.—Add 5 c.c. of concentrated hydrochloric acid and 0.5 c.c. of 1 per cent. alcoholic solution of naphthoresorcinol to 5 c.c. of urine. Boil the solution for about a minute. It darkens markedly. Cool and extract by shaking with a few c.c. of ether. The supernatant ethereal layer acquires a reddish-violet or violet-blue colour, while in pentosuria the ether becomes yellow or red.

This test was regarded as a specific one for glycuronates; but Neuberg has obtained results which indicate that its specific character is doubtful. Neuberg has therefore modified the test in the following way :—

Place 10 c.c. of the fresh urine in a small separating funnel, add 2 c.c. of dilute sulphuric acid (sp. gr. 1.16), 10 c.c. of ethyl-alcohol and 20 c.c. of ether. Mix thoroughly and add a little saturated solution of sodium chloride to facilitate the separation of the ether. Run off the subjacent aqueous alcoholic layer, then shake up the ethereal solution with 2 or 3 c.c. of saturated sodium chloride solution, and finally separate the ethereal solution as completely as possible. Filter the ethereal solution through a small dry filter paper into a porcelain basin, add 5 c.c. of water, and evaporate the ether on the water bath. Divide the aqueous solution into two portions and

apply the orcinol and naphthoresorcinol tests. Both tests give a positive result. The colour reaction yielded by orcinol was a reddish-green, but the characteristic absorption band between C and D was well defined.

This method is based on the fact that the conjugated glycuronates are soluble in a mixture of alcohol and ether, while the pentoses being insoluble in ether remain in the aqueous layer. The test is so delicate that conjugated glycuronates may be detected in normal urine by this method.

VI. ACETO-ACETIC ACID.

This acid is frequently found in diabetic urine.

TESTS.

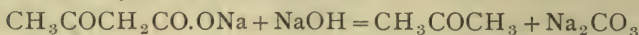
1. *Add to a small quantity of the urine, ferric chloride, as long as a precipitate of ferric phosphate continues to form. Filter, and add a little more solution of ferric chloride. A violet-red colour appears when aceto-acetic acid is present.*

Certain drugs (*e.g.* salicylates, salicylurates, and antipyrine), which are sometimes present in the urine, give a similar colour reaction. None of these substances are decomposed by boiling the urine, and the colour reactions given by them with ferric chloride are therefore obtained after the urine has been boiled, while aceto-acetic acid is decomposed by boiling into acetone and carbon dioxide. Urine containing aceto-acetic acid therefore no longer gives the ferric chloride reaction after it has been boiled and cooled. The decomposition of aceto-acetic acid is frequently incomplete if the period of boiling be brief. In order to ensure complete removal of aceto-acetic acid (and the products of its decomposition), it is, therefore, advisable to concentrate the urine on the water bath to about a third of its bulk, cool, and then apply the ferric chloride test. If the originally positive reaction be now negative, then the colour reaction is due to aceto-acetic acid, while one or other of the drugs mentioned is the cause of the colour reaction if the reaction be still positive.

2. *Acidify the urine with dilute sulphuric acid, and extract with ether. Separate the ether, and shake it with a very dilute solution of ferric chloride. The aqueous solution acquires a violet-red tint.*

3. *On heating with dilute caustic alkali, aceto-acetic acid yields acetone, and the carbonate of the alkali used.*

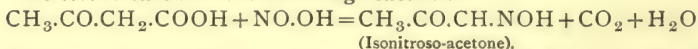
The acetone may be detected by its odour, or distilled off and tested for in the distillate.



An artificial urine, giving these three reactions, may be prepared by the addition of ethyl aceto-acetate or preferably sodium aceto-acetate to normal urine.

4. HURTLEY'S TEST.—Add 2.5 c.c. of concentrated hydrochloric acid, and 1 c.c. of 1 per cent. solution of sodium nitrite to 10 c.c. of a dilute solution of aceto-acetic acid, or of urine containing that acid. Allow the solution to stand for 2 minutes, add 15 c.c. of strong ammonia and 5 c.c. of a 10 per cent. solution of ferrous sulphate or chloride, shake thoroughly, transfer to a Nessler glass and allow the mixture to stand for some hours. The precipitate of ferrous hydrate should not be filtered off. A violet or purple colour slowly develops at a rate which varies with the amount of aceto-acetic acid present. If a larger quantity of nitrite be used, the colour obtained with the ethyl ester is blue. The reaction can be obtained at a dilution of 1 in 50,000. By comparing the intensity of the colour reaction with that given by solutions of sodium aceto-acetate of known strength, the quantity of aceto-acetic acid present may be approximately determined.

The test is based on the following reactions :—



The isonitroso-acetone then forms a violet-coloured ferrous salt with ferrous hydrate $[(\text{CH}_3\text{CO.CH:NO})_2\text{Fe}]$.

Note.—Preparation of sodium aceto-acetate from the ethyl ester (Hurtley).—Add 100 c.c. of N/1 caustic soda to 13 grms. of ethyl aceto-acetate placed in a 500 c.c. flask, dilute with water to 500 c.c., and allow to stand for 44.5 hours. The ester is almost completely saponified.

VII. ACETONE.

Use a dilute solution of acetone for the following tests :—

1. To a portion of the solution add a few drops of caustic soda, and then some solution of iodine in potassium iodide. The fluid becomes turbid, and gives an odour of iodoform. Allow the yellowish-white precipitate to settle, and examine the deposit microscopically.

2. LEGAL'S TEST.

Add to a portion of the solution a few drops of freshly prepared aqueous solution of sodium nitroprusside, then

render alkaline with NaOH = a deep red colour. Acidify with acetic acid. The colour darkens to a violet.

Ammonia may be used for this test instead of caustic soda. The red colour develops more slowly in the presence of ammonia than in that of caustic soda. (Cf. Creatinine, p. 218).

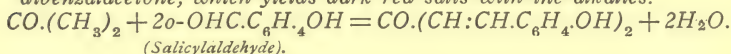
Compare with Weyl's test for creatinine. The tests may be applied directly to the urine, or to the first 20 c.c. of the distillate from 250 c.c. of urine acidified with dilute hydrochloric acid.

3. ROTHERA'S TEST.—To 5 c.c. urine add sufficient finely powdered ammonium sulphate to saturate the solution, then 2 to 3 drops of a fresh 5 per cent. solution of sodium nitroprusside and 1 to 2 drops of strong ammonia. A positive reaction is indicated by the development of a characteristic permanganate colour. "The delicacy of the reaction for acetone in urine reaches 1 in 20,000. The reaction must not be declared negative until after half an hour has been allowed for the development of colour."

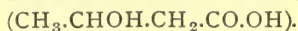
This test is a more delicate one for aceto-acetic acid than for acetone (Hurtley). 1 part of aceto-acetic acid in 100,000 gives the reaction in 2 minutes, 1 in 400,000 in 5 minutes.

4. Salicylaldehyde reaction (Fabinyi and Frommer).—Add 1 c.c. of a 10 per cent. solution of salicylaldehyde in alcohol to 5 c.c. of the urine, mix, and then add 1 gm. of solid caustic soda or potash. Set aside the solution without shaking. A dark red colour develops at the surface of the solid alkali.

Under the influence of alkali, the salicylaldehyde unites with the acetone to form a product of condensation known as o-o-dioxydibenzalacetone, which yields dark red salts with the alkalis.

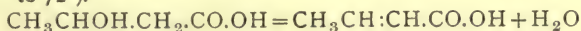


VIII. β -HYDROXYBUTYRIC ACID



β -Hydroxybutyric acid is usually obtained as an odourless, colourless, transparent, non-volatile syrup. When pure it can be obtained as highly hygroscopic plate-like crystals, which melt at 49° to 50° . It is laevorotatory ($[\alpha]_D^{20} = -24.12^\circ$ for concentrations under 12 per cent.—Magnus-Levy). It is readily soluble in water, alcohol, and ether. It is a monobasic acid yielding salts, which are all readily soluble in water, as a rule sparingly soluble in alcohol and insoluble in ether. It is not precipitated by the addition of basic lead acetate or basic lead acetate plus ammonia.

When heated with dilute sulphuric acid it yields α -crotonic acid (m.p. 71° to 72°).



When oxidised with hydrogen peroxide, or potassium dichromate and sulphuric acid, it yields aceto-acetic acid and acetone.

BLACK'S TEST.—Concentrate 20 c.c. of urine in a porcelain basin on the water bath to one-fourth of its original bulk, acidify with a few drops of hydrochloric acid, and mix the fluid with sufficient plaster of Paris to form a thick paste. Allow the paste to stand until it begins to "set," then break up with a stirring rod, and extract twice with 10 c.c. portions of ether. Decant the ethereal extracts into another porcelain basin, evaporate the ether on the water bath, dissolve the residue in 5 c.c. of water, and neutralise the aqueous solution with a little barium carbonate. Then add to the cooled and filtered solution 2 to 3 drops of hydrogen peroxide ("10 volumes per cent."), mix thoroughly, and add a few drops of Black's reagent (5 grms. of ferric chloride and 0.4 gm. of ferrous chloride dissolved in 100 c.c. of water). The typical red colour due to the formation of the ferric compound of aceto-acetic acid gradually develops, reaches its maximal intensity, and then gradually fades. 1 part of β -oxybutyric acid in 10,000 parts of urine can be detected by this method.

β -Hydroxybutyric acid has been found in the urine in a number of pathological conditions. Large quantities (15 grms. or more per day) have been found in cases of severe diabetes.

IX. Unknown substances—(1) For Ehrlich's diazo-reaction the reader is referred to works on clinical diagnosis, or to Neubauer-Huppert's "Analyse des Harns" (pp. 1252-1256).

(2) For Cammidge's reaction, see *Lancet*, 1904, i. p. 782, and 1905, ii. p. 14. The precise nature of the different substances which yield these reactions is still uncertain.

URINARY SEDIMENTS

These may be classified into—(1) Organised, and (2) unorganised. The best method of separation is by means of the centrifuge. Text-books of clinical diagnosis should be consulted for details with regard to the examination of organised deposits.

UNORGANISED SEDIMENTS.

Take the reaction of the urine prior to microscopic examination of the sediment.

If ACID, the following substances may be present, either—

(1) In an amorphous form, or (2) in a crystalline form.

I. FROM ACID URINE.

1. Amorphous.

(a) URATES.

This most common deposit is met with, both in concen-

trated normal and in pathological urines (*e.g.* febrile). It is a yellowish-brown to brick-red deposit. Usually it is referred to as the "brick-dust" deposit. The sediment consists of small granules, frequently accompanied by crystals of *calcium oxalate* and of *uric acid*. The small spherules consist of the biurates of sodium and potassium, possibly also of ammonium and any other bases present. Frequently very fine needle-like crystals adhere to the spherules.

Remove a small quantity of the sediment and boil with a little water. It dissolves. Acidify the hot fluid with hydrochloric acid. Cool, and examine microscopically the sediment which forms = pigmented crystals of uric acid. The deposit also gives the murexide reaction.

(b) CALCIUM OXALATE.

The sediment appears in the form of dumb-bell shaped or spheroidal bodies. These are insoluble in acetic acid, readily soluble in hydrochloric acid.

(c) BILIRUBIN or HÆMATOIDIN.

Amorphous yellow granular masses which give Gmelin's reaction.

2. Crystalline Sediment.

(a) URIC ACID (*see* p. 209). Some of the more common crystalline forms are represented in figure (Plate I.). The deposit is often termed the "cayenne pepper" deposit.

(b) BILIRUBIN or HÆMATOIDIN.

Small yellow rhombic plates, which give Gmelin's reaction.

(c) CALCIUM OXALATE.

Colourless, transparent, highly refractive, octahedral crystals (envelope-shaped), insoluble in acetic acid, readily soluble in hydrochloric acid.

(d) AMMONIUM MAGNESIUM PHOSPHATE (only in very faintly acid urine).

These crystals are in the form of large knife-rests, which are soluble in acetic acid. Some of the smaller crystals resemble those of calcium oxalate, but are readily distinguished by their solubility in dilute acetic acid.

The following sediments are rare :—

(e) CYSTINE.

Regular hexagonal plates, soluble in ammonia, insoluble in acetic acid.

(f) CALCIUM HYDROGEN PHOSPHATE ($\text{CaHPO}_4 + 2\text{H}_2\text{O}$).

Large prismatic crystals, frequently arranged in rosettes. Add a solution of ammonium carbonate. The crystals are eaten into, and ultimately break down into an amorphous deposit. They dissolve readily in dilute acetic acid.

(g) TYROSINE.

Star-shaped bundles of fine needles, insoluble in acetic acid, soluble in ammonia and hydrochloric acid. (See pp. 123-124 for tests.)

II. IN ALKALINE URINE.

If the urine be alkaline, the following sediments may occur :—

I. Amorphous.

(a) EARTHY PHOSPHATES.

Fine granules, dissolving in dilute acetic acid without evolution of carbon dioxide ($\text{Ca}_3(\text{PO}_4)_2$ and $\text{Mg}_3(\text{PO}_4)_2$).

(b) CALCIUM CARBONATE.

In two forms—(a) Fine granules, soluble with effervescence in dilute acetic acid. (β) Dumb-bell shaped, or spheroidal masses, frequently showing concentric striation. They dissolve readily in dilute acetic acid with evolution of carbon dioxide. This sediment is very rare in human urine.

(c) ACID AMMONIUM URATE.

Pigmented spherules, frequently with small crystals, "hedgehog spines," adhering. They first dissolve in hydrochloric acid, and then rhombic crystals of uric acid separate out.

2. Crystalline.

AMMONIUM MAGNESIUM PHOSPHATE.

Large colourless prisms of knife-rest or coffin-lid shape. These are readily obtained in the sediment and scum which form in a urine undergoing ammoniacal fermentation.

(For some of the more important urinary crystals, see Plate I.)



SECTION II
QUANTITATIVE



CHAPTER XVI

ESTIMATION OF SOME OF THE ELEMENTS IN ORGANIC COMPOUNDS

THE methods mentioned in this chapter are those most frequently used in physiological chemistry. The reader is referred to text-books of practical organic chemistry for the methods used in the estimation of carbon and hydrogen, Dumas' method for nitrogen, and Carius' method as applied to the estimation of sulphur, phosphorus, and the halogens.

Kjeldahl's method for the estimation of organic nitrogen, and methods for the determination of organic sulphur are described in the chapter on urine.

I. One of the most useful methods of analysing organic substances is that of Neumann.

PRINCIPLE OF NEUMANN'S METHOD.

The organic material is oxidised with a mixture of equal volumes of nitric (sp. gr. 1.4) and concentrated sulphuric acids. When oxidation is completed, the solution obtained, after dilution with water, may be examined either qualitatively or quantitatively for all non-volatile bases, and acids with the exception of the constituents of the oxidising mixture. The method is specially useful for the determination of organic phosphorus. With certain modifications the method can also be used for the estimation of organic chlorine.

PRELIMINARY PREPARATION OF THE MATERIAL FOR ANALYSIS.

As a rule, the method can be directly applied to the analysis of most solutions of organic material or to organic solids. When the solution only contains a trace of the element to be estimated (*e.g.* iron in urine), a large quantity of the solution (500 c.c.) is acidified with one-tenth its volume of nitric acid, and gradually added from a separating funnel with long bent stem to 30 c.c. of nitric acid (sp. gr. 1.4) kept boiling in a Kjeldahl flask, the rate of addition being adjusted so as approximately to keep pace with evaporation (Neumann, "Arch. f. Anat. u. Physiol.," 1902, p. 132). When the fluid has been concentrated to a volume of about

50 c.c. or less, the oxidation is completed in the way described below.

Preparation of the oxidising reagent.—Add gradually (mixing thoroughly after each addition) 100 c.c. of concentrated sulphuric acid to 100 c.c. concentrated nitric acid (sp. gr. 1.4).

The oxidation.—Add 10 c.c. of the oxidising reagent to 5 c.c. of the solution, or to 0.2 to 0.5 grm. of the organic solid placed in a 750 c.c. Kjeldahl flask. The quantity of solution or solid chosen for analysis varies according to the approximate percentage of the element to be determined. If much organic material be present, e.g. in a diabetic urine, allow the mixture to stand for a short time at room temperature in the draught chamber, then heat gradually with a small flame. Dark brown fumes consisting mainly of nitrogen peroxide are evolved. When the evolution of gas begins to lessen, add drop by drop from a separating funnel (supported in a glass or porcelain ring) with long stem so bent that the stopcock can be manipulated without exposing the hand to the direct action of the acid vapours, more of the reagent to the boiling fluid until the quantity of brown vapours again lessens. Then cease adding the reagent, but continue to boil the contents of the flask. When nearly all the nitric acid has been driven off, the residual sulphuric acid solution may be colourless, yellow, dark brown or even black. If the fluid be practically colourless the oxidation is completed. If it be brown or black, more of the oxidising reagent, or (if a considerable quantity of the latter has already been added) pure nitric acid must be added. Allow the fluid to cool somewhat before adding more of the reagent. The latter precaution is necessary because the temperature of the contents of the flask is now approximately that of boiling concentrated sulphuric acid, and the addition of either nitric acid, or a mixture containing it, would cause a violent evolution of oxides of nitrogen. Gradually add either nitric acid or the oxidising reagent as before, again heat the solution until the evolution of NO_2 slackens, cease the addition of nitric acid or reagent and continue the heating until the greater part of the nitric acid has been driven off. The residual solution should now be colourless or very faintly yellow. If still brown, the nitric acid must again be added as before. Not more than 40 c.c. of the reagent should be used for one analysis. If difficulty be experienced in completing the oxidation with this amount, add pure nitric acid in the later stages instead of the mixed acids.

Allow the contents of the flask to cool, then add 2 to 3 times as much water as the total quantity of oxidising reagent used, and boil for 5 to 10 minutes. NO_2 derived from the decomposition of nitrosylsulphuric acid is evolved. The colourless solution, thus obtained, may be used for the determination of phosphorus, iron, calcium, magnesium, potassium, or sodium.

1. Estimation of Phosphorus.—The quantity of substance taken for analysis should not contain more than 0.05 grm. of phosphorus expressed in terms of P_2O_5 (0.2 to 0.3 grm. of sodium nucleate from the thymus, or 5 to 10 c.c. of urine may be used).

REAGENTS REQUIRED.

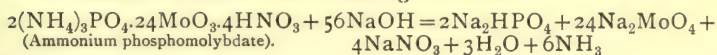
- (a) 50 per cent. ammonium nitrate solution.
 (b) 10 per cent. ammonium molybdate (prepared by dissolving the finely-powdered salt at room temperature and filtering).
 (c) N/2 caustic soda. (d) N/2 sulphuric acid.
 (e) 1 per cent. alcoholic solution of phenolphthalein.

After oxidation is complete, and the solution has been diluted and boiled as already described, quantitatively transfer the contents of the Kjeldahl flask to a litre flask marked with a "chinagraph" pencil to indicate approximately a volume of 150 c.c. Wash out the Kjeldahl flask repeatedly with distilled water, transferring the washings to the second flask until the marked level is reached. Add 50 c.c. of (a), heat to 70°-80°, then add 40 c.c. of (b), shake vigorously for half a minute and allow the yellow precipitate of ammonium phosphomolybdate to settle for 15 minutes. Decant part of the fluid from the precipitate and filter through ash free filter paper (about 5 to 6 cm. radius) previously moistened with cold water. The flask may be conveniently supported in an oblique position on a retort ring fixed slightly above the level of the filtering funnel. By inclining the neck of the flask (after slightly greasing the under side of the lip with a thin film of vaseline) the clear supernatant fluid is allowed to flow into the filter paper in a continuous gentle stream at a rate corresponding to that of filtration. In this way very little of the precipitate is transferred to the filter paper. The latter should never be more than two-thirds full. When the precipitate in the flask has been drained as far as possible, wash down the neck of the flask with about 100 c.c. of cold water, shake thoroughly, allow the precipitate to settle and repeat the decantation and filtration of the supernatant fluid. The washing with water is repeated 3 or 4 times until the filtrate is no longer acid to litmus.

Transfer the filter paper to the flask, fill up to the 150 c.c. mark with water, shake thoroughly, and add from a burette an accurately measured quantity of N/2 caustic soda, until a clear colourless solution is obtained. Then add a further 6 c.c. of N/2NaOH, and boil the fluid until ammonia is no longer evolved (testing with moistened red litmus paper). Cool the solution thoroughly under the tap, add 6 to 8 drops of (e), and titrate with N/2 sulphuric acid until the red colour is just discharged. Deduct the number of c.c. of N/2H₂SO₄ (= b) from the number of c.c. of N/2NaOH originally added (= a), the result (= a - b) when multiplied by 1.268 gives the quantity in milligrammes of phosphorus as P₂O₅, or when multiplied by 0.553 gives the quantity in mgs. of phosphorus as such.

PRINCIPLE OF METHOD.

The method is based on the following reaction :—



56 mols NaOH are therefore equivalent to 1 mol P₂O₅.

The total phosphorus (inorganic and organic) is estimated by the foregoing method. The inorganic phosphorus, *e.g.* of urine, may be determined by simply adding 10 c.c. of the oxidising agent to 5 c.c. of urine previously diluted with about 50 c.c. of water. The solution is kept cool under the tap and made up to 150 c.c. with water. Ammonium nitrate solution is added, and the further stages of the method are the same as those already described.

When carried out with due care the method gives very accurate results. For class work a solution of chemically pure phosphate (*e.g.* $\text{Na}_2\text{HPO}_4 + 12\text{H}_2\text{O}$. m.w. 358, or $\text{Na.NH}_4\text{HPO}_4 + 4\text{H}_2\text{O}$. m.w. 210) of known strength, to which cane sugar has been added, may be given out for analysis. The technique of the method has been improved by other observers. The chief aim of the various modifications has been to facilitate filtration by filtering under pressure (see Plimmer and Bayliss, "Journal of Physiology," vol. xxxiii. p. 44).

2. **Organic Chlorine.**—With certain modifications, Neumann has applied his method of oxidation to the estimation of inorganic and organic chlorine. Plimmer has proved that prussic acid is evolved along with HCl during the oxidation of protein solutions, and that certain modifications have to be introduced in the method in order to secure accurate results (Plimmer, "Journal of Physiology," vol. xxxi. p. 65).

3. Estimation of the Iron in Blood (Neumann-Hanslian).

Completely oxidise about 10 grms. of blood (defibrinated or whole blood) by the method already described.

REAGENTS REQUIRED.

(a) Ferric chloride solution containing .002 gm. iron in 10 c.c. (prepared by diluting 20 c.c. of Fresenius' ferric chloride solution and 2 c.c. of concentrated hydrochloric acid up to a litre with water).

(b) N/250 sodium thiosulphate solution (approximate). Prepare by dissolving 1 gm. of sodium thiosulphate and 1 gm. of ammonium carbonate in water and making up to a litre.

(c) Starch solution. Dissolve 1 gm. of soluble starch in 500 c.c. of water, and boil for 10 minutes.

(d) Zinc reagent. Dissolve 25 grms. of zinc sulphate and 100 grms. of sodium phosphate separately in water, then mix the solutions in a litre flask, just dissolve the precipitate of zinc phosphate by the gradual addition of dilute sulphuric acid, and make up the solution to a litre with water.

All the reagents [(b) to (d)] and the sulphuric and nitric acids must be free from iron. A blank experiment may be carried out with the reagents to ascertain that they are free from iron.

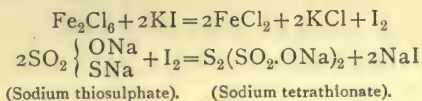
(1) THE PRINCIPLE OF THE METHOD.

When a solution of zinc phosphate in slight excess of ammonia is heated, a precipitate of ammonium zinc phosphate forms, which carries

down all the iron. The ferric phosphate is then filtered off, washed, dissolved in dilute hydrochloric acid, and the iron present estimated by titration.

(2) **Titration of the sodium thiosulphate solution.**

Since N/250 sodium thiosulphate is unstable, it requires to be standardised by titration with the standard solution of iron. The following equations represent the reactions on which the titration method is based :—



Place 20 c.c. of (a) (= .004 grm. Fe) in 100 c.c. stoppered flask, add 30 c.c. of sodium chloride solution (56.6 grms. of pure NaCl dissolved in water and made up to a litre), and 5 c.c. of (c), completely displace the air in the flask by passing through it a stream of CO_2 , add 3 grms. of potassium iodide, stopper the flask, shake thoroughly, and allow to stand at room temperature for 20 minutes. Then titrate the solution with (b) until the blue colour is just discharged, pass a stream of CO_2 through the flask for a few minutes, then stopper and allow to stand for 2 to 3 minutes. If the solution remains colourless the titration is complete. If not, decolorise by the addition of sodium thiosulphate and repeat the foregoing procedure. The number of c.c. of sodium thiosulphate added is equivalent to .004 grm. of iron.

(3) **The estimation of the iron in the product of incineration.**

Dilute the solution with 3 volumes of water, boil for at least 10 minutes, cool, and add 20 c.c. of (d). Then gradually add ammonia until a permanent precipitate of zinc phosphate forms (cooling the flask occasionally under the water tap). Just dissolve the precipitate by the gradual addition of dilute ammonia (1:4). Then boil the solution for at least 10 minutes until a permanent precipitate separates. Allow the precipitate to settle, decant and filter the supernatant fluid through ash free filter paper (8 cm.). Test a few drops of the filtrate for iron with dilute HCl and potassium thiocyanate. If the reaction be positive, replace the filtrate in the flask, and again heat the solution. The precipitate in the flask should be washed at least 3 times by decantation. The filtrate from the final washing must give no reaction with potassium iodide and starch (absence of nitrites). If the latter reaction be positive the washing must be continued.

Insert the filtering funnel into the neck of the Kjeldahl flask, and wash with 100 c.c. hot dilute hydrochloric acid (20 c.c. of the concentrated acid made up to 100 c.c. with water). Heat the Kjeldahl flask on the water bath for about 10 minutes, and filter the cooled contents into a 250 c.c. measuring flask. Wash out the Kjeldahl flask repeatedly with hot water, cool, and filter the washings into the measuring flask until the total filtrate amounts to about 200 c.c. All the iron is now in solution as ferric chloride. Before titration the solution

should be only faintly acid. Gradually add caustic soda solution (about 20 per cent.) until a trace of zinc phosphate separates. Then dissolve the precipitate with a few drops of dilute HCl (1:4), and make up the solution to 250 c.c. with water.

A 50 c.c. portion of the fluid is transferred with a pipette to a bottle provided with a glass stopper, and the amount of iron determined by titration in the way described in (2), the addition of sodium chloride being omitted (see Neumann, l.c., p. 362, and Hanslian Abderhalden's "Biochemische Arbeitsmethoden," vol. vi. p. 376).

II. Estimation of the ash remaining after the dry incineration of an organic substance or mixture of substances such as occurs in the tissues of the body.

Dry the organic material to constant weight at 110° to 115°, or preferably in a vacuum oven at 80° to 90°. Place 1 or 2 grms. of the dried material in an accurately weighed platinum or porcelain crucible (with lid), and determine the exact quantity of material by reweighing the platinum crucible with its contents, using an analytical balance weighing to .0001 or .0002 gm. Place the crucible obliquely on a triangle of platinum wire, or of quartz, partially cover the crucible with its lid and heat gently over a small flame until the substance chars. It is advisable to heat as slowly as possible, ultimately for a short time to a dull red in order to prevent loss by volatilisation of sodium or potassium chloride. Allow the charred mass to cool, extract the residue at least five times with boiling water, and filter the washings through a small ash-free filter paper (about 5 cm.) into a beaker. Gradually transfer the filtrate in small portions to the platinum crucible and evaporate to dryness on the water bath, taking care that the beaker is thoroughly washed out. Place the filter paper with extracted residue in the platinum crucible, and dry at 115° to 120°. Then complete the incineration by heating gradually with a small bunsen flame (or spirit lamp).

The oxidation of the carbon may be greatly facilitated by moistening the cooled, charred residue (obtained in the first stages of the method) with a few c.c. of pure 3 per cent. hydrogen peroxide solution, slowly evaporating to dryness on the sand bath or on an asbestos plate, and finally completing the incineration by heating to a dull red. The hydrogen peroxide solution must be free from inorganic salts, and therefore must leave no residue when a small quantity is evaporated to dryness.

When all the carbon has been oxidised, allow the crucible to cool in the exsiccator and weigh.

Weight of crucible and organic material — weight of crucible = xg

Weight of crucible and ash — weight of crucible = Kg

Percentage of ash in the organic material is therefore $\frac{100 K}{x} g.$

Neumann's method of "wet incineration" has the advantage of rendering impossible any loss of sodium or potassium such as may occur if the temperature rise too high during a dry incineration.

CHAPTER XVII

SOME PHYSICAL CHEMICAL METHODS EMPLOYED IN BIOLOGICAL CHEMISTRY

CONTENTS

- I. Determination of the freezing points (Cryoscopy) and allied measurable properties of solutions.
- II. Determination of the reaction of solutions.
- III. Determination of surface tension.
- IV. Determination of viscosity.

I.—Determination of the Depression of the Freezing Point of Water produced by the addition of Solutes.

THIS determination involves the measurement of small differences in temperature. The most essential part of the apparatus used is therefore the thermometer, which must fulfil the following requirements :—(1) It must be graduated so that the temperature can be accurately read to 0.001° . (2) It is desirable that the thermometer should be applicable to the estimation of the depression of freezing point of other solvents than water produced by known concentrations of solutes, *e.g.* benzene, freezing point, $+5.4^{\circ}$; glacial acetic, $+17^{\circ}$; phenol, $+40^{\circ}$, etc. Obviously, a thermometer graduated to read to $.001^{\circ}$ for a range of 40° to 50° would be of such an unwieldy length as to be practically unusable. Beckmann overcame this difficulty by devising a differential thermometer having a large bulb, so constructed that the amount of mercury in the bulb can be varied.

The scale of the Beckmann thermometer usually extends over 6° , divided into tenths and hundredths. By means of a lens the level of the mercury in the capillary can be read to $.001^{\circ}$. There are no fixed points on the scale, such as 0° , since only differences of temperature are to be measured. At the

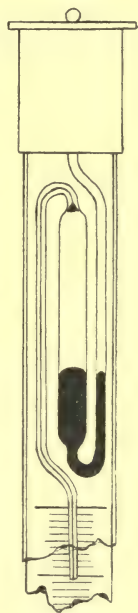


Fig. 1.

opposite end from the bulb the fine capillary of the thermometer is bent downwards and opens into a reservoir (fig. 1), which contains any excess of mercury. If the thermometer is to be "set" for a higher temperature, mercury is transferred from the capillary to the reservoir; whereas, if it is to be set for a lower temperature, mercury is transferred from the reservoir to the capillary. In accurate work a correction in the reading has to be made to compensate for the varying amount of mercury in the bulb. (For this and other corrections see W. Watson's "Practical Physics," pp. 260 to 264, or text-books of practical physical chemistry.)

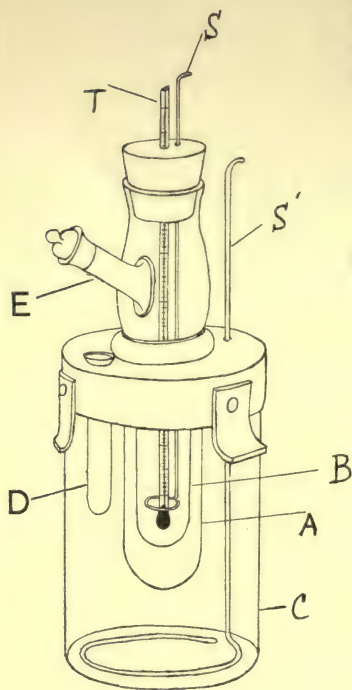


Fig. 2.

The thermometer is set for water as the solvent in the following way:—The bulb is placed for some minutes in a mixture of ice and water at 0° . If the upper limit of the mercury in the capillary stands within one degree of the top of the scale, the thermometer is already "set." If the mercury level lies near the bottom of the scale, more mercury must be introduced. Remove the thermometer from the water, and allow the mercury to rise until it fills the capillary and upper part of the reservoir, then invert the thermometer and tap gently so as to cause a small quantity of the mercury in the lower part of the reservoir to unite with that in the upper part. Now place the thermometer again erect in the vertical position. Immerse the bulb in a water bath, and gradually reduce the temperature of the water to $+2^{\circ}$ (the temperature of the bath being determined with an ordinary thermometer) by adding small pieces of ice. Keep the Beckmann's thermometer immersed in water at 2° for several minutes. Then remove the excess of mercury from the upper part of the reservoir to the lower by gentle tapping, at the same time holding the thermometer fixed in the vertical position with bulb still immersed in water at 2° . Immerse the bulb of the thermometer again in water, and gradually lower the temperature of the water to 0° by surrounding it with a freezing mixture. After a few minutes the level of the mercury in the capillary should now remain constant within 1° of the top of the scale. The actual adjustment is simple and easily learned, although the description of the method of procedure is somewhat tedious. The thermometer should be set 24 hours before it is used for an estimation.

The additional apparatus required (fig. 2) consists of an outer thick-

walled glass jar (*C*) containing the freezing mixture, having a temperature 4° to 6° below the freezing point of the solvent (*e.g.* finely powdered ice and a saturated solution of sodium chloride). A large thick-walled tube (*A*) is inserted through the cover of this vessel. A narrower tube (*B*), with stoppered side tube (*E*), is fitted into this by means of a rubber collar, or ground-glass junction, the space between the two tubes serving as an air jacket separating the freezing tube (*B*) from the freezing mixture, and thus ensuring a slow rate of cooling. *B* is closed with a rubber, or better vulcanite, stopper, through a central opening in which the Beckmann thermometer (*T*) passes, while a platinum or glass stirrer (*S*) passes through a small lateral hole. The thermometer may be fixed at any desired level by means of a rubber collar.

SOME APPLICATIONS OF THE METHOD.

1. Determination of the molecular weight of urea.

Measure 20 c.c. of water with an accurate pipette into the tube *B*. An alternative more accurate method is to weigh the quantity of water introduced. Solvents other than water should always be weighed to an accuracy of .01 gm.

Insert the thermometer, taking care that the bulb does not come in contact with the sides or bottom of the tube (*B*). As the mercury sinks keep stirring the water in the tube (*B*). The mercury continues to sink (say to 4.95°) then suddenly rises until it reaches a fixed point (say 5.35°). Supercooling therefore = 0.40° . Tap the thermometer with the finger, and take the reading when the level of the mercury has become constant. Vigorous stirring is important since it lessens the tendency to supercooling. Remove the tube (*B*) with the thermometer, allow the ice to melt, then again insert (*B*) into (*A*), and repeat the observation. The two results should agree to within $.002^{\circ}$.

Remove the tube (*B*) with thermometer, allow the ice to melt, withdraw the stopper of the side tube (*E*), and introduce a weighed quantity of urea (about 0.2), care being taken that the urea falls direct into the solvent. This is most conveniently done if the urea be weighed in a long narrow weighing tube, which can readily pass through the side tube (*E*). The weight of urea is accurately determined by difference thus:—

Weighing bottle + urea	4.7382 grms.
Weighing bottle . . .	4.5260

Weight of urea . . . 0.2122 gm.

Dissolve the urea in the water by stirring, replace tube (*B*), and determine the freezing point of the solution in the same way as that of the solvent. The mercury falls as before (say to 4.60), then rises to a maximum (say 5.02), at which it remains constant for a short time, and then slowly falls. If the supercooling has not exceeded 0.5° , the maximal temperature corresponds to the freezing point of the solution. The depression of freezing point, or difference between

the freezing point, of the solution and that of the solvent = $5.35 - 5.02 = 0.33^\circ$.

If supercooling has exceeded 0.5° , it may be prevented in a second estimation in the following way:—

Place a glass rod, slightly moistened with the solvent, in the test-tube D. A film of solidified solvent soon forms on its surface. When the mercury in the Beckmann thermometer has fallen a little below 5.02 , withdraw the stopper from the side tube, raise the stirrer, touch its surface with the cold end of the glass rod, withdraw the rod, stopper the side tube, and renew vigorous stirring.

If a further quantity of urea (about 0.2 grm.) be added to the solution, and Δ again determined, the value will now be found to be about 0.66 .

This result indicates that for a given solute and solvent, Δ is proportional to the percentage concentration of solute (Blagden, 1788). On the other hand, if another solute, *e.g.* glucose, be selected, it will be found that 3.00 grms. of glucose dissolved in 100 grms. of water produce about the same lowering of the freezing point as 1.00 grms. of urea in the same quantity of solvent. Raoult was the first observer to establish the truth of a more general law which may be expressed thus:—Solutions containing the same number of mols of different solutes dissolved in a fixed quantity of the same solvent lower the freezing point of the solvent to the same extent.

The law, which holds only for dilute solutions of substances that do not dissociate into ions, may be formulated thus:—If T represents the freezing point of solvent, and T_1 that of a solution containing p.g. of a solute having the molecular weight (M) dissolved in 100 grms. of solvent, the molecular depression of the freezing point is a constant (C).

$$\frac{M(T - T_1)}{p} = C.$$

Obviously, if 1000 grms. of solvent be selected instead of 100 grms., the constant will be equal to $0.1 C$. The value of C varies for different solvents. It is 18.5 for water, 50 for benzene, 73 for phenol, and 39 for glacial acetic acid.

The value of the constant may also be calculated from the latent heat of fusion of the solvent (l) by means of the following formula (Van't Hoff):—

$$C = \frac{.0199T^2}{l}$$

T is the freezing point of the pure solvent in absolute measure.

By means of the first formula, the molecular weight of urea may be calculated:—

$$\begin{aligned} p &= 0.2122 \times 5 = 1.061 \text{ g} \\ T - T_1 \text{ or } \Delta &= 0.33 \\ \therefore 0.33M &= 18.5 \times 1.061 \\ M &= 59.5 \text{ (true value } 60) \end{aligned}$$

In the determination of molecular weights of solutes, the following formula, which may be readily derived from the foregoing one, is usually employed :—

$$M = \frac{100 Cw}{\Delta W}$$

M = unknown molecular weight of solute,
w = weight of solute. W = weight of solvent.

Δ = observed depression of freezing point. C = "the molecular depression" of the solvent.

As a rule, a high degree of accuracy is not required in the determination of molecular weights by the freezing point method. In some cases, (e.g. in determining whether a given sugar has the molecular weight of a pentose or hexose), a considerable degree of accuracy is essential.

The usual error of observation is about 5 per cent. By making a correction for supercooling, the error can be reduced to about 1 per cent.

NOTE.—Correction for supercooling :—"If c is the specific heat, w the weight of the solution, l the latent heat of the solvent, θ° the supercooling, and x the weight of solvent which solidifies, we have—

$$x = \frac{cw\theta^\circ}{l}$$

Hence when working out the strength of the solution we must deduct the quantity x from the weight of solvent in order to obtain the strength of the solution which has the observed freezing point" (W. Watson, *loc. cit.*, p. 260). For water as solvent, c is 1, and l is 80.

2. **Abnormal molecular weights** are frequently found by the method just described. (1) Too high molecular weights are found when association of the solute molecules has occurred, and (2) too low molecular weights are found when the solute molecules have undergone dissociation.

Association.—The tendency to the formation of molecular aggregates varies both with the nature of the solvent and the solute. It is least marked in the case of solvents, such as water, which have high dielectric constants;¹ while the formation of complex molecules readily occurs in such solvents as benzene and chloroform which have low dielectric constants. Such solutes as amino-acids and proteins have so marked a tendency to the formation of molecular aggregates that the dissociating action of water may be partially overcome.

Dissociation.—Since water is the chief solvent² present in protoplasm, dissociation of solutes plays a more important rôle in physiological processes than association, and our practical work will therefore be confined to the consideration of dissociation.

Arrhenius was the first observer to give a satisfactory explanation of

¹ For definition and significance see text-books of Physical Chemistry, and of Physics.

² The solvent power of lipins and fats must also be remembered.

the abnormal behaviour of certain solutes. He pointed out that the solutes, which yield too small molecular weights, whatever the method of measurement employed, all conduct the electric current, and attributed their abnormal behaviour to the fact that they dissociate more or less completely into ions. The fraction of the solute, which dissociates into ions, increases with dilution, and also varies according to the nature of the solute. Soluble salts, acids, and bases all dissociate more or less completely into ions; while neutral indifferent solutes such as the sugars, urea, etc., do not dissociate and therefore yield normal molecular weights.

Determination of the degree of Dissociation of Sodium Chloride in a solution containing 9 grms. of the Salt dissolved in 1000 grms. of Water.

Determine the depression of freezing point of this solution in the way already described. The depression of the freezing point of the solvent was found to be -0.55° .

We have already seen that $\Delta = \frac{1.85w}{M}$. w is the weight of substance in grms. dissolved in 1000 grms. of water, and M is the normal molecular weight of the solute on the assumption that no dissociation takes place. Since the molecular weight of sodium chloride is 58.5, the normal depression of freezing point (which would be found if no dissociation occurred) can readily be calculated thus:—

$$\Delta = \frac{1.85 \times 9}{58.5} = -0.285^{\circ}.$$

The degree of dissociation of the sodium chloride, in the solution examined, may be calculated from the observed depression in the following way:—

$\frac{w}{M}$ represents the number of mols (or gramme molecules), or fraction of a mol present in 1000 grms. of solvent. Let x represent this number, which is the number of molecules of sodium chloride originally present—no dissociation being assumed. Let α represent the fraction of sodium chloride which undergoes dissociation into n ions. The number of undissociated molecules is therefore $x - \alpha x$. The number of ions resulting from the dissociation is $n\alpha x$, and the total number of particles in solution, i.e. the sum of the molecules and ions is $x - \alpha x + n\alpha x$ or $x[1 + \alpha(n - 1)]$. n is 2 in the case of sodium chloride which dissociates into Na^+ and Cl^- . Therefore on substituting these values in the original formula, one obtains the following result:—

$$\begin{aligned} 0.55 &= 1.85x(1 + \alpha) = \frac{1.85w(1 + \alpha)}{M} = \frac{1.85 \times 9(1 + \alpha)}{58.5} \\ \therefore \alpha &= \frac{58.5 \times 0.55}{1.85 \times 9} - 1 = 0.93 \end{aligned}$$

93 per cent. of the salt has therefore undergone dissociation into ions.¹

¹ This value is too high, probably owing to insufficient accuracy in the experimental determination.

The value of α at any dilution can also be calculated from determinations of molecular conductivity. The reader is referred to text-books of physical chemistry for information regarding the latter measurements.

Determinations of the Freezing Points of the Fluids of the Body, e.g. Blood and Urine.—These are carried out in the way already described. The average depression for human blood is -0.56° . It is very constant in health—a variation of $.01^\circ$ to $.02^\circ$ being pathological. We have already seen that blood contains a large quantity of proteins in addition to smaller quantities of crystalloids consisting of electrolytes, such as sodium chloride, and non-electrolytes, such as urea and glucose. The depression of freezing point is almost entirely accounted for by the crystalloids present. This result therefore justifies the classification of the solutes in such a fluid as blood into two main groups—crystalloids, and substances of such high molecular weight that they exert a practically inappreciable effect on the freezing point of the solvent. The latter group consists of the emulsoid colloids—proteins. For further information regarding the colloids the reader is referred to the literature quoted in Chapter IV.

The freezing point of urine varies from -0.50° to -2.24° . The normal freezing point cannot be very definitely fixed (about -1.70°). The freezing point of urine multiplied by the daily quantity of urine in c.c. measures the total number of molecules and ions excreted per day ("molecular diuresis"). This value divided by the body weight gives a ratio termed "*Diurèse moléculaire totale*" by Claude and Balthazard.

Calculation of the Osmotic Pressure of a Solution from the Depression of the Freezing Point.—Van't Hoff proved that in dilute solutions the osmotic pressure has the same value as the pressure which the solute would exert in the gaseous state if it occupied the same volume as the solution (or solvent) at the same temperature. Osmotic pressures are, therefore, subject to the same laws as gaseous pressures. The mol or gramme molecule of any substance, which does not dissociate, occupies a volume of 22.4 litres at 0°C. , and under a pressure of one atmosphere (760 mm. of mercury). Consequently, according to Boyle's law, a mol of any substance, which does not dissociate, when occupying a volume of 1000 c.c. exerts a pressure of 22.4 atmospheres at 0° . A solution of this concentration gives a depression of the freezing point of water of 1.85° .

Further, the lowering of the freezing point has been proved to be proportional to the osmotic pressure. Therefore—

$\frac{p}{22.4} = \frac{\Delta}{1.85}$. p is the unknown osmotic pressure in atmospheres of the solution. Δ is the corresponding depression of the freezing point.

Consequently,
$$p = \frac{22.4\Delta}{1.85}.$$

Obviously, the osmotic pressure of a solution may also be stated in

mm. of mercury by multiplying the foregoing result by 760. Calculate the osmotic pressure of normal human blood (result, 6.78 atmospheres or 6.78×760 mm. of mercury). Calculate in the same way the osmotic pressure of 0.9 per cent. sodium chloride and of normal urine ($\Delta = -1.7^\circ$).

The reader is referred to works on physical chemistry for direct methods of determining osmotic pressure, and for molecular weight determinations based on the lowering of vapour pressures, and elevation of the boiling points of solvents by dissolved substances.

G. Barger's microscopic method for the estimation of molecular weights and osmotic pressures is described in the "Journal Chem. Soc.," vol. lxxxv. p. 286, and in his article in Abderhalden's "Handbuch der biochem. Arbeitsmethoden," vol. viii. pp. 1-11. The special value of this method for biological chemistry depends on the fact that a few centigrams of a solid or a few drops of a liquid suffice for a determination.

II.—The Hydrogen ion Concentration of the Body Fluids

As the reaction of the fluids of the body is of extreme importance in many biological processes, such as the regulation of enzyme action, the interchange between the tissue cells and the surrounding medium, etc., it is necessary to be able to determine at least approximately the hydrogen ion, or hydron, concentration, as this is a measure of the "true" reaction. The "true" acidity or alkalinity of a solution is not the full acid or alkali value as estimated by titration, but is determined by the existing hydron concentration (cH^+). The cH varies with the degree of dissociation of the acid or alkali as well as with their respective concentrations. The greater the degree of dissociation of the acid for a given total concentration, the higher the hydron and the lower the hydroxyl ion concentration, the converse holding for the dissociation of the base. Thus, for example, a decinormal solution of hydrochloric acid, being approximately 90 % dissociated, the H^+ and Cl^- concentrations are not .1 but .09 normal, and in the same way a .1 n. NaOH with 84 % dissociation is .084 n. as regards Na^+ and OH^- . On the other hand, weak acids and bases show for a given concentration a much smaller degree of dissociation, .1 n. acetic acid being only .0013 n. as regards H^+ and CH_3COO^- , and .1 n. ammonium hydrate .0014 n. NH_4^+ and OH^- . In order to understand the significance of certain terms used in connection with hydron concentration values, it is necessary to give the following explanatory statement. In all aqueous fluids there is a small amount of the water in the dissociated state as H^+ and OH^- , equilibrium being established between the dissociated ions and the undissociated water molecules in obedience to the law of mass action, so that—

$$\frac{cH^+ \times cOH^-}{cH_2O} = \text{constant.}$$

As the concentration of the undissociated water molecules is so great, it may be regarded as equivalent to unity and constant, so that the

product of the ionic concentrations in a fixed volume, 1 litre, and at a fixed temperature, 18° C., may be regarded as the constant, namely, the ionic product constant. Stating the ionic concentrations as gramme equivalent concentrations per litre, this product has the following value at 18° in all aqueous fluids, the gramme ionic concentrations being denoted by the square brackets—

$$[H^+] \times [OH^-] = .72 \times 10^{-14},$$

or stating this value in decadic logarithmic form $\bar{15.8573}$ or in the notation suggested by Sørensen $10^{-14.14}$, where the minus sign governs both the index and the fraction.

If, then, the concentration of one of the ions be determined in any aqueous fluid, that of the other can be directly arrived at, and as it is usually the $[H^+]$ which is estimated, the $[OH^-] = \frac{10^{-14.14}}{[H^+]}$.

In "true" neutral solutions $[H^+] = [OH^-]$, and therefore each is $10^{-7.07}$,¹ in acid solutions $[H^+] > [OH^-]$, and is, therefore, greater than $10^{-7.07}$, while in alkaline solution $[H^+] < [OH^-]$, and is, therefore, less than $10^{-7.07}$. An increase therefore in the negative exponent denotes a fall in $[H^+]$, and a decrease a rise in the same. As all hydron concentrations dealt with are below normality, the power is always a negative power of ten, and for convenience in notation Sørensen takes the reciprocal of this power and so omits the minus sign, and denotes this pH. Thus in reading from a pH value to the actual concentration value, one transforms the former into the ordinary decadic logarithmic form and consults an antilog. table.

For example, pH 6.82 = $\bar{7.18}$ =

$$cH + 1.514 \times 10^{-7} \text{ normal}$$

and the solution would be faintly acid.

Regarding then the reaction of the body fluids from the standpoint of their hydron concentration, it is interesting to note the range of values covered by the more important secretions, etc., stating these

¹ In order to understand clearly the significance of such a statement as that the hydron concentration of pure water at 18° is $10^{-7.07}$, and that this is really the expression of the normality of the dissociated hydrogen ions, the following explanation may be of value. If one litre of pure conductivity water were introduced between platinum electrodes placed 1 cm. apart, the resistance at 18° C. would be approximately 24,038 ohms, or the reciprocal of this, the conductivity, would be 4.16×10^{-5} reciprocal ohms. If the litre of water had contained one gramme equivalent H^+ and one gramme equivalent OH^- , its conductivity would then have been 489 reciprocal ohms, and the solution would have been a normal one as regards these ions. As the pure water, however, only possesses a conductivity of 4.16×10^{-5} (4.16×10^{-7}) the normality as regards H^+ and OH^- is

$$\frac{4.16 \times 10^{-7}}{489} = .85 \times 10^{-7}$$

$$\log .85 \times 10^{-7} = \bar{8.93}$$

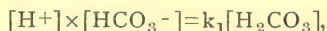
or pH (and pOH) 7.07

In other words, one gramme of hydrogen ions and 17 grammes of hydroxyl ions are present in rather less than 12 million litres of water.

values directly as cH^+ (in terms of normality) and also in pH notation.

	cH^+	pH
Gastric juice : normal	1.7×10^{-2}	1.77
hyperacidity	5×10^{-2}	1.52
Pancreatic juice	$1-2 \times 10^{-8}$	8-7.7
Intestinal juice	2×10^{-8}	7.7
Fæces (variable)	1×10^{-8}	8
Milk (cow's)	2.7×10^{-7}	6.57
„ (human)	1.07×10^{-7}	6.97
Urine (variable)	1×10^{-5} to 1×10^{-7}	5-7
Blood	4 to 7×10^{-8}	7.35-7.5

These values are naturally subject to variations, in certain cases slight, as in blood, in others great as in urine. Those fluids which maintain a fairly constant reaction, even when acid or basic radicles enter or leave them, owe this property very largely to the presence of regulator constituents, so-called buffer substances. When pure water or water containing such neutral salts as sodium chloride is exposed even to the air of the room, the hydron concentration rises rapidly above the neutral point value owing to the absorption of carbonic acid, so that the pH may be 6 or reach even a more acid value. Although such a fluid as blood may readily absorb or give off carbonic acid, the reaction is only comparatively slightly affected, owing mainly to the presence of the salt of this weak acid, bicarbonate, in moderate concentration. In all solutions containing free carbonic acid in solution, whether there be base present or not, equilibrium is established between $[H^+]$ and $[HCO_3^-]$ and undissociated $[H_2CO_3]$ as is expressed in the following equation :—



in which k_1 , the first dissociation constant of H_2CO_3 , has the value 3×10^{-7} . If the $[HCO_3^-]$ of such a solution is raised by the addition of a salt which on dissociation furnishes this anion, in order that the value of k_1 should remain constant the $[H^+]$ must fall, or, in other words, the dissociation of the H_2CO_3 must be pushed back. It is evident, therefore, from the equation

$$[H^+] = k_1 \frac{[H_2CO_3]}{[HCO_3^-]}$$

that with a rise in the value of the denominator the $[H^+]$ must fall and that the higher the $[HCO_3^-]$ the less effect will a given H_2CO_3 rise produce on the original hydron concentration. In this way acetates lower the “true” acidity of acetic acid solutions, and the same holds for the salts of other weak acids. Mixtures of mono- and di-basic phosphates act in a similar way, their reaction being determined by the ratio of the

one salt to the other. The second dissociation constant of phosphoric acid is concerned in this equilibrium equation

$$[H^+] \times [HPO_4^-] = k_2 [H_2PO_4^-] \quad k_2 = 1.95 \times 10^{-7}$$

In all mixtures of mono- and di-basic phosphates

$$[H^+] = 1.95 \times 10^{-7} \frac{[H_2PO_4^-]}{[HPO_4^-]}$$

The value of $[H_2PO_4^-]$ is determined by the concentration of NaH_2PO_4 and the degree to which this salt dissociates into its constituent ions, while the value of the $[HPO_4^-]$ is fixed in the same way by the di-basic phosphate. So long as in the mixture there are present both mono- and di-basic phosphates the addition of acid or alkali produces only slight disturbances in the hydrion concentration compared to those which would occur in water or in sodium chloride solutions.

METHODS FOR DETERMINATION OF THE HYDROGEN ION CONCENTRATION.

Two methods have been mainly employed.

(1) The Indicator or Colorimetric method.

(2) The Electrometric method.

The former is the more readily carried out, and, in many cases, will furnish the necessary information regarding the true reaction of a solution, although frequently not with the same accuracy as the latter method.

1. The Indicator Method.

According to the older view indicators were regarded as weak electrolytes in which the undissociated part and the ions were of different colours or only one of the two was coloured. Thus, in the case of a weak indicator acid like p. nitrophenol, the transition colour zone would lie between the colourless undissociated acid molecule and the yellow-coloured anion of the dissociated salt. The change point or better transition zone, for different indicators occurs throughout a wide range of cH^+ and is determined by the dissociation constant of the indicator acid or base. Thus, the hydrion concentration of the middle of the transition zone of p. nitrophenol corresponds to the point when there is an equal concentration of the undissociated colourless acid and the yellow dissociated anion of the salt of the acid. At this point the $[H^+] =$ dissociation constant of p. nitrophenol.

$$\frac{[H^+] \times [p. \text{nitrophenolate}^-]}{[p. \text{nitrophenol}]} = k$$

and in the middle of the transition zone

$$[H^+] = k \frac{[.5 \text{ p. nitrophenol}]}{[.5 \text{ p. nitrophenolate}^-]} \quad \text{or} \quad [H^+] = k$$

In the case of all indicators, whether they obey the ordinary law of

dissociation and act in the way above described (the older view), or if the change in colour in the transition zone be due to an alteration in the constitution of the molecule, a tautomeric change (the more recent view), the alteration is always a function of the hydrion concentration. In order that the indicator should be a satisfactory one, its transition zone should not be much affected by alterations in temperature, saline concentration, or the presence of such organic substances as occur in the body fluids. The colour change should also be practically instantaneous and should be readily perceptible. During recent years a large and increasing number of indicators has been made use of, so that within such a wide range of reaction as from pH 1 to pH 14 the particular hydrion concentration can be defined with comparative ease and rapidity.

For convenience in class work the solutions will be taken in groups, beginning with those of greater acidity. The ordinary test tube stand may be employed tilted backwards to an angle of about 45° . It is more convenient, however, to have stands with clips to fix the tubes, and behind the stand there should be pinned a sheet of white paper. When slightly coloured or turbid fluids require to be examined, some form of colorimeter, preferably of the Walpole type,¹ should be used. The principle of this method depends upon the comparison between (a) a tube of the coloured fluid placed above the standard solution plus indicator, and (b) the coloured fluid plus indicator over a tube of water.

So-called comparators, blackened boxes with places for the tubes, and having longitudinal or circular openings through which the coloured solutions may be examined according to the Walpole principle, have been largely made use of (Hurwitz and others, Cole and Onslow). With these comparators ground glass or colour screens may be used. There are certain disadvantages in the examination of tubes placed one row behind the other. It is more satisfactory to use small bottles with flat polished sides such as are employed in spectroscopic work.

Clark and Lubs (1916) have introduced a most convenient series of standard solutions, gradually rising by steps of .2 pH from 1.2 to 10 pH. These are HCl—KCl mixtures (.02 molar) for pH 1.2 to 2.2, acid potassium phthalate—HCl solutions for 2.2 to 3.8 pH, acid potassium phthalate—KOH solutions for 4 to 6.2 pH, and phosphate and borate mixtures up to 10 pH. These tables, along with a note on the method of preparation, are given on pp. 388, 389. In the text, however, for convenience in class work, this range is covered by solutions which are in general use in all laboratories.

SOLUTIONS, pH 1 TO 3.

Take three test-tubes, into the first put 10 c.c. .1 n. HCl, into the second 10 c.c. .02 n. HCl, and into the third 10 c.c. .1 n. CH₃COOH.

¹ "Proc. Physiol. Soc." and "Biochemical Journal," 1910.

Add to each 5 drops of the indicator, thymol blue (.04 per cent.).

Tube (1) shows deep pink colour.

Tube (2) shows fainter pink colour.

Tube (3) shows yellow with faint orange tint.

As the transition colour zone of this indicator (in its acid range) lies between pH 1.2 and pH 2.8 and the colour change from the former to the latter pH is red→yellow, it is evident that (1) is on the more acid side of the zone, (3) at the other extreme, and (2) intermediate.

The theoretical pH values are (1) 1.04, (2) 1.72, (3) 2.89, and these values may be arrived at by considering the degrees of dissociation of the three acid solutions. Thus, if we take from conductivity determinations:—

(1) to be 91 per cent. dissociated its normality in terms of

$[H^+]$ is 9.1×10^{-2} or pH 1.04;

(2) calculated for 95 per cent. dissociation would be

1.9×10^{-2} or pH 1.72; while

(3) if 1.3 per cent. dissociated would be

1.3×10^{-3} or pH 2.89.

If now the solutions be tested in the same way with the indicator brom-phenol blue, the transition colour zone of which lies between pH 2.9→4.5, it will be found that all give a yellow tint showing that (yellow→blue),

even the decinormal acetic acid lies on the acid side of the colour change zone of this indicator.

Specimens of gastric juice give a colour change with thymol blue similar to .02 N. HCl.

SOLUTIONS, pH 3 TO 4.

Suitable standard solutions of this range may be made from decinormal acetic acid and decinormal sodium acetate mixtures, or acid phthalate mixtures (p. 388).

Methyl orange pH 3→4.4 and brom-phenol blue pH 2.8→4.6 may be used as indicators.
(red→yellow) (yellow→blue)

Prepare mixtures of decinormal acetic acid and decinormal sodium acetate with the acid and the salt in the following proportions:—

(a) $\frac{16}{1}$ (acetic) (acetate) pH 3.54.

(b) $\frac{4}{1}$ (acetic) (acetate) pH 4.14.

Take 4 tubes, labelling them (1), (2), (3), and (4). Into (1) and (3) put 10 c.c. of (a), and into (2) and (4) 10 cc. of (b). Add 5 drops of methyl-orange (.1 per cent. in 50 per cent. alcohol) to (1) and (2), and 5 drops of brom-phenol blue (.04 per cent. in water) to (3) and

(4). (1) shows a deep orange, (2) a very faint orange, (3) a yellowish-grey with blue tint, (4) a fairly deep blue.

These solutions may be used as standards for comparison with gastric contents of low acidity.

SOLUTIONS, pH 4 TO 5.

Prepare acetic-acetate mixtures of the following proportions:—

$$(a) \frac{2}{1} \frac{(\text{acetic})}{(\text{acetate})} \text{ pH } 4.44.$$

$$(b) \frac{1}{1} \frac{(\text{acetic})}{(\text{acetate})} \text{ pH } 4.74.$$

$$(c) \frac{1}{2} \frac{(\text{acetic})}{(\text{acetate})} \text{ pH } 5.0.$$

For this range methyl-red (.02 per cent. in 50 per cent. alcohol) pH 4.5 \rightarrow 5.5 may be used.

(red \rightarrow yellow)

Take 10 c.c. samples of (a), (b), and (c), and add 5 drops of the indicator to each one.

(a) shows deep pink.

(b) faint pink.

(c) orange with faint pink tinge.

The most acid urines have reactions corresponding to (c). In the titration backwards with acid of Na_2HPO_4 to NaH_2PO_4 (see p. 297), the pH of the latter corresponds approximately to (a), or slightly to the acid side of (a), so that the change of methyl orange from yellow to pink may be used for this end-point.

SOLUTIONS, pH 5.3 TO 6.3.

This range can be satisfactorily obtained from acetic acid acetate mixtures in which the acetate concentration is gradually raised. The phthalate NaOH mixtures (p. 388) may also be used. Urine may be examined as the test fluid, and the acetate mixtures as standards.

Prepare the following standard mixtures:—

$$(a) \frac{1}{4} \frac{(\text{acetic})}{(\text{acetate})} \text{ pH } 5.35.$$

$$(b) \frac{1}{8} \frac{(\text{acetic})}{(\text{acetate})} \text{ pH } 5.65.$$

$$(c) \frac{1}{32} \frac{(\text{acetic})}{(\text{acetate})} \text{ pH } 6.25.$$

Brom-cresol purple (.04 per cent. in water), transition zone pH 5.3 \rightarrow 6.5 is a satisfactory indicator for this range. Take 10 c.c. (yellow \rightarrow purple)

of (a), (b), and (c) in labelled test-tubes, and add to each 5 drops of the indicator.

(a) is yellow in tint.

(b) an impure yellow with purple tinge.

(c) violet.

Compare the reaction of the early morning urine with a portion passed about two hours later (after breakfast), the former will probably give a slight deepening of the normal yellow colour of the urine, the latter a violet or purple indicating the greater acidity of the morning urine and the so-called "alkaline tide" of the later urine.

SOLUTIONS, pH 6 TO 7.

This range, up to the neutral point, includes among biological fluids the less acid urines and milk. The whey from milk clotted with neutral rennin powder may be used for testing reaction against the standards. Phosphate mixtures are convenient as standards for this range and the next one. These may be readily prepared by the method described by Prideaux.¹

With a standard pipette run into a 100 c.c. flask 10 c.c. molar phosphoric acid, then from a burette run in the number of c.c. normal NaOH mentioned below, and make up with water to 100 c.c. By gradually increasing the number of c.c. NaOHn. from 10 c.c. to 20 c.c., one obtains the range from NaH_2PO_4 to Na_2HPO_4 .

The following standard mixtures should be made:—

(a) 12 c.c. NaOHn. pH 6.0.

(b) 14.5 c.c. „ pH 6.5.

(c) 16.55 c.c. „ pH 6.9.

Two indicators should be used for this range, brom-cresol purple with the range already mentioned and brom-thymol blue (0.4 per cent. in water), pH 6→7.6.

(yellow→blue).

Take two sets (A and B) of three tubes (a), (b), (c), and add to one set (A) 5 drops of the former indicator, and to the other (B) 5 drops of the latter indicator.

A. (a) yellow with slight violet tint.

(b) weak purple.

(c) deep purple.

B. (a) yellow.

(b) green with faint blue tinge.

(c) blue with faint green tinge.

Take tubes with specimens of urine passed during the day, and another set with whey from clotted milk. Add the abovementioned indicators and compare the tints with those given by the standards.

The extreme limits of this range should also be defined by the use of

¹ "Journal, Chem. Soc. Trans.," 1911, p. 1225.

methy! red for the more acid (giving a yellow colour) and phenol red for the neutral end of the range (giving a yellow colour).

SOLUTIONS, pH 7 TO 8.

This is an extremely important range, including, as it does, blood and allied fluids, as also the pancreatic and intestinal juices. Suitable standards may be made from the phosphate mixtures by continuing the series referred to in the preceding section.

(a)	NaOHn.	17.6 c.c.	pH 7.12.
(b)	" "	18.6 "	" 7.3.
(c)	" "	19 "	" 7.5.
(d)	" "	19.5 "	" 8.

Three indicators may be used for this range—

Brom-thymol blue,	.	.	.	pH 6→7.6 (yellow→blue),
Phenol red,	.	.	.	pH 6.8→8 (yellow→red),
Neutral red,	.	.	.	pH 6.8→8 (red→yellow).

As the reaction of the blood is determined by the ratio of H_2CO_3 to NaHCO_3 for the reasons given previously (p. 266), it is of interest to study, in the first place, the change in reaction which bicarbonate solutions of different concentration undergo when brought into equilibrium with the carbonic acid of the expired air.

Take 6 tubes and label them (1)-(6).

Into (1) and (2) put 10 c.c. .003 n. NaHCO_3 .

Into (3) and (4) put 10 c.c. .03 n. NaHCO_3 .

Into (5) and (6) put 10 c.c. .3 n. NaHCO_3 .

(2), (4), and (6) should then be brought into equilibrium with the carbonic acid of expired air by breathing slowly through each solution one full expiration. This may be simply done by blowing through a short length of rubber tubing attached to a pipette dipping into the solution. Add 5 drops of brom-thymol-blue to each of the six tubes. The tubes (1), (3), and (5), which have not been breathed through, show practically the same blue colour, while those breathed through, (2), (4), and (6) show a distinct difference in tint. Number (2) is yellow, (4) green with slight blue tinge, (6) practically unaltered blue. That is to say, for approximately the same H_2CO_3 concentration, the cH has been raised most (to the acid side of the neutral point) in the weakest and least in the strongest bicarbonate solution.

A second similar set of six tubes treated in the same way may now be tested with another indicator. Add 5 drops of phenol red solution (.02 per cent.) to each tube. Of those which have not been breathed through (1), (3), and (5), (1) is evidently the least alkaline as is

shown by the faint yellow tinge in the red which is absent from (3) and (5). This is due to the small amount of dissolved carbonic acid producing a more marked rise in the cH of the weak bicarbonate than in that of the stronger solutions. The pink tints of (3) and (5) correspond to the alkaline side of the transition zone of this indicator.

Of the tubes which have been breathed through, (2) is yellow, (4) a faint orange, and (6) red, practically unaltered by the expired air. The change in (2) is to the acid side of the neutral point, (4) slightly on the alkaline side, and (6) markedly on the alkaline side of the neutral point.

From the physiological standpoint the .03 n. NaHCO_3 is the most important of the three solutions, as the shift in reaction produced by breathing through it gives rise to a pH similar to that of blood exposed to the same carbonic acid pressure. The change in reaction in the case of .003 n. NaHCO_3 is much greater, and in the case of .3 n. NaHCO_3 much less than that which occurs in blood plasma treated in the same way. Neutral red may be used in place of phenol red, having approximately the same transition zone as the latter, only the colour change as the pH rises is pink \rightarrow yellow, the reverse of phenol red. Standard phosphate solutions of a range from pH 6 to pH 8 should be used for comparison.

Phosphate Mixtures.	Brom-thymol Blue.	Phenol Red.
pH.		
7.12	Blue with Green Tint.	Yellow with faint Pink Tinge.
7.3	Increasing depth	Increasing depth
7.5	of Blue.	of Red.
8.0	↓	↓

These standards (or similar ones) should be used for comparison when determining colorimetrically the reaction of such fluids as blood plasma, lymph, cerebro-spinal fluid or the intestinal secretions, as also the reaction of the most alkaline urines.

In order to obtain an approximate value for the reaction of blood plasma, take 5 c.c. .9 per cent. NaCl to which 5 drops of phenol red have been added, and now with a pipette, the point of which is at the bottom of the tube, allow 5 c.c. of plasma to run underneath the saline solution. The supernatant fluid at the surface of contact with the blood plasma shows an orange tint. The same may be done for comparison with 5 c.c. of phosphate solutions of different pH, and the results compared with those obtained in the case of the plasma.

2. The Electrometric Method.

The hydron concentration values of practically all aqueous solutions, standard or otherwise, can be determined with great accuracy by this

method. The colorimetric method by indicators is based upon the results obtained by the electrometric one, and hence it is of great importance to gain even a general idea of the principles upon which the latter method is based. Although an equipment is required which is not always obtainable, a brief description of the method may be of value even to those who may confine themselves to the colorimetric one in their practical work. In the case of most standard solutions, the hydrion value can be arrived at theoretically from a knowledge of the dissociation constants of the weak acids or bases, and the degrees of dissociation of their salts. The electrometric values may be therefore compared with the theoretical ones as well as with the results obtained from the use of indicators. Apart from practical text-books in physical chemistry there are excellent descriptions of the method as applied to biochemical problems in the works of Sørensen, Michaelis, and Mansfield Clark.¹ These works would naturally be consulted by all employed in electrometric work of this kind.

PRINCIPLE OF THE METHOD.

According to Nernst, when a metal such as silver is immersed in a solution containing silver ions in low concentration, there is a difference of potential established between the metal and the solution, the degree of which is dependent upon the concentration of the Ag^+ in the solution. The silver plate tends to give off silver ions with a positive charge, and giving a negative charge to the plate, an electrical double layer is thus produced. The measure of this so-called electrolytic solution tension is the osmotic pressure of the Ag^+ in solution, which just prevents the discharge of positively charged silver ions from the plate, and thus leads to a disappearance of the electrical double layer. If the electrolytic solution tension be denoted as P and the osmotic pressure of the corresponding ion p , then there are three conditions to be considered—

- (1) $P=p$ No double layer and no p.d.
- (2) $P>p$ Metal - charge, solution +
- (3) $P<p$ Metal + charge, solution -

In all cases where the metal is pure the p.d. is dependent upon the concentration of the corresponding ion in the solution. The determination of the p.d. of such a single electrode is, however, unsuitable, but if a chain or cell be formed with two silver plates of the same purity immersed each in a solution containing Ag^+ but different in concentration, there will be a definite electromotive force in the circuit which can be determined by connecting up the cell with a potentiometer. This arrangement constitutes a concentration cell. It is found that, in all cases where the ion (in this case Ag^+) is univalent, the e.m.f. is .057 volt at 18° when one solution has ten times the ionic concentration of the other, the direction of the current being from the dilute to the more concentrated solution.

¹ Sørensen, "Ergebnisse der Physiologie," xii., 1912; Michaelis, "Die Wasserstoff ion en Konzentration;" W. Mansfield Clark, "Determination of Hydrogen Ions," 1920.

An example of such a cell would be two silver electrodes in 0.1 n. and 0.01 n. Ag^+ solution respectively. If the ion were of greater valency (n), for a similar difference in concentration the e.m.f. would be $\frac{.0577}{n}$ volt. Stated generally if c = conc. of Ag^+ in the stronger solution and c^1 = conc. of Ag^+ in the weaker, then at 18°

$$\text{e.m.f.} = .0577 \log_{10} \frac{c}{c^1}$$

so that in the special case above described

$$\text{e.m.f.} = .0577 \log_{10} \frac{.1}{.01} = .0577 \text{ volt.}$$

If one concentration be known—*e.g.* c , the ratio of c to c^1 can be determined from the e.m.f., and so the concentration of c^1 can be calculated.

The estimation of the concentration of the hydrogen ion depends upon the same principle. In this case a hydrogen electrode requires to be formed, and this is accomplished by charging a platinum black deposit on a platinum wire or plate with pure hydrogen. Just as differences in concentration of the metal, zinc, or silver (as, for example, by the use of amalgams) will alter the electrolytic solution tension of the electrode (P), so will differences in the hydrogen concentration in the hydrogen electrode alter the solution tension of the hydrogen plate. Hence when the sole variable requires to be the concentration of the hydrogen ions in solution, it is necessary that the hydrogen pressure in the hydrogen electrode be fixed, that is pure hydrogen at atmospheric pressure. This being fixed, the e.m.f. of two hydrogen electrodes connected up in the same way as the silver ones will obey the same law as the latter, the hydrogen ion being also univalent. An example will make this clear. Two hydrogen electrodes are connected up, one containing decinormal HCl , and the other decinormal NaOH . The former being 91 % dissociated (p. 269), the hydrion concentration is .091 n = c . What is the hydrion concentration of the decinormal NaOH = c^1 ? After correction for the contact difference of potential where the two fluids meet, the e.m.f. was found to be .6951 volt at 18° .

$$.6951 = .0577 \log_{10} \frac{c}{c^1}$$

$$\therefore \frac{c}{c^1} = 10^{12.045}$$

$$\begin{aligned} \therefore c^1 &= c \times 10^{-12.045} \\ &\text{or } .091 \times 10^{-12.045} \\ &= .83 \times 10^{-13} \\ &\text{or } 10^{-13.08} \end{aligned}$$

That is to say, .1 n. NaOH has a $\text{pH} = 13.08$.

The OH^- concentration can be readily obtained from this—

$$\text{as } [\text{OH}^-] = \frac{k(\text{H}_2\text{O})}{[\text{H}^+]} = \frac{10^{-14.14}}{10^{-13.08}} = 10^{-1.06}.$$

In this case a hydrogen electrode with a known hydrion concentration is

taken as the standard from which the unknown hydron concentration of the other gas electrode is calculated, but it is more convenient to use as the standard a calomel electrode. This consists of a vessel containing a layer of mercury at the bottom with which a platinum wire makes contact, above this there is a layer of calomel mercury paste, and above this a KCl solution of a definite concentration (saturated, normal, or decinormal). When the e.m.f. of a chain consisting of a calomel electrode and a hydrogen electrode of unknown hydron concentration is determined, it requires to be corrected so that the e.m.f. may be stated in terms of the system—hydrogen electrode of normal cH, and the hydrogen electrode of unknown cH in order that the latter may be calculated from the equation given above. This is done by subtracting the e.m.f. of a calomel and normal hydrogen electrode chain from that of the same calomel electrode connected up with the hydrogen electrode of unknown hydron concentration so that the equation becomes e.m.f. of calomel el. and hydrogen el. of unknown cH(c^1) minus e.m.f. of calomel el. and normal hydrogen electrode ($c=1$)

$$=.0577 \log_{10} \frac{c}{c_1} = .0577 \log_{10} \frac{1}{c_1}.$$

As the e.m.f. of the decinormal calomel electrode plus n. hydrogen electrode at 18° is .3377 volt, then

$$\text{e.m.f. of unknown chain} - .3377 = .0577 \log_{10} \frac{1}{c_1}$$

$$\therefore \log_{10} \frac{1}{c_1} \text{ (or pH)} = \frac{\text{estimated e.m.f.} - .3377}{.0577}$$

For example, if the estimated e.m.f. were .5108 volt, then

$$\text{pH} = \frac{.5108 - .3377}{.0577} = 3$$

or $\log_{10} c^1 = 10^{-3}$ or .001 n. hydron concentration.

Although the chief source of the e.m.f. is at the junction of the plate and the solution, apart from the negligible development at metallic junctions in the circuit, there may be, and often is, a considerable e.m.f. at the surface of contact between the fluids in the two electrodes. This is considerable in certain cases, for example, where the cH is moderately high owing to the much greater velocity of this ion than the associated electro-negative ion, but in the presence of salts in fairly high concentration the contact or diffusion potential becomes much less, and can be usually eliminated by the use of 3.5 normal KCl as the connecting solution.

MEASUREMENT OF THE P.D. IN THE CALOMEL ELECTRODE/HYDROGEN ELECTRODE CHAIN.

The method usually employed is a compensation or zero method (potentiometer method). The two ends of a uniform wire, stretched along a divided scale, are connected with the opposite poles of one or two accumulators, the e.m.f. of which is greater than that of the system

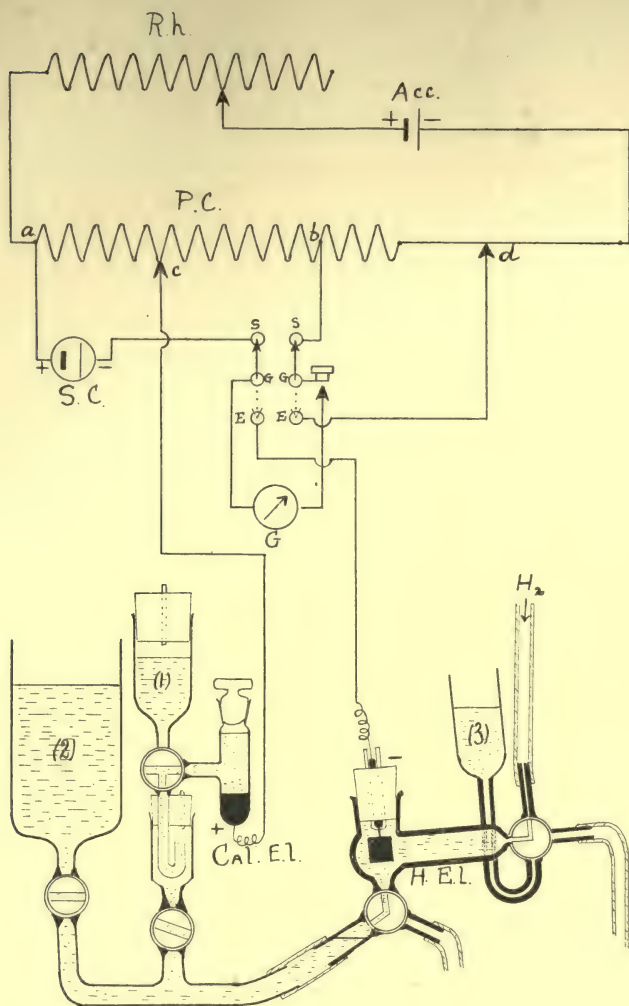


Fig 3.

The various parts are lettered as follows:—Acc.=1 or 2 storage cells, Rh=adjustable rheostat, P.C.=potential coils, a . . . b=standard cell's p.d. with fixed contacts, c . . . d=p.d. of cell to be measured (with movable contacts), S.C.=standard cell, SS=switch between standard cell and galvanometer, EE=switch between electrodes and galvanometer, GG=g galvanometer connections, G=g galvanometer, Cal.=calomel electrode .1 n., H.El=hydrogen electrode, (1)=.1 n. KCl, (2)=3.5 n., KCl for connecting system, (3)=solution for hydrogen electrode, H_2 =hydrogen gas inlet.

to be measured. The double electrode system, the e.m.f. of which is to be measured, is connected up with the stretched wire so that the calomel electrode (+) is attached to the same end of the wire as that to which the + pole of the accumulator is connected, while the hydrogen electrode (-) is in connection with the sliding contact, a fairly high resistance galvanometer or capillary electrometer being inserted in this circuit. The sliding contact is moved along the wire until the balance point is reached and the galvanometer or electrometer indicates the zero, and the length of wire (a) so tapped off noted. The cell of unknown e.m.f. (x) is now replaced by a standard cadmium cell of known e.m.f. (y), the balance point again determined and length of wire noted (b). The lengths of wire tapped off (a and b) are proportional to the e.m.f. of the

unknown (x) and known (y) cells, $\frac{x}{y} = \frac{a}{b}$, $x = \frac{ay}{b}$. Owing to alterations in

the accumulator the potentiometer current is subject to variations which make it necessary to determine anew the fall in potential along the wire. This is an inconvenient and time wasting procedure, and so arrangements require to be made so that by altering the resistance in the accumulator circuit there is always the same uniform current passing between the standard cell contacts. Convenient forms of potentiometer of the direct reading type are obtainable in which the stretched wire is replaced by resistance coils (potential coils on calibration) and finally slide wire (fig. 3). By adjusting the resistance in the rheostat (Rh) the fall in potential across each of the coil sections is .1 volt and along the 100 cm. slide wire also .1 volt so that each cm. division corresponds to 1 millivolt, this being again subdivided to allow of a reading to .1 or .2 of a millivolt. The general arrangement of one type of such a direct reading potentiometer (Leeds and Northrup) is shown in fig. 3, with standard cell and calomel / hydrogen electrode chain connected up.

This calomel / hydrogen electrode system is the one used by W. Mansfield Clark. There are many modifications of the arrangement figured, but the general principle of all of them is the same. As accurate regulation of temperature is requisite in this as in other physico-chemical methods, the wiring arrangements of lamps for temperature control of hot air, oil or water baths are shown in fig. 4.¹ The arrangement has been found a most useful one in this laboratory. The long radiator lamp is inserted into the bath along with the vertical bulb of the toluene-mercury regulator. For many purposes a large glass tank loosely placed in a wooden frame and with a suitable stirring arrangement is extremely useful, as, for example, in conductivity, viscosity, and dialysis experiments.

The wiring arrangements of the thermo-regulator are evident from the figure. The large lamp is a 220 volt radiator lamp, the small one a 50 volt 16 c.p. carbon lamp, Rh=adjustable rheostat, R=relay, and T.R.=toluene mercury regulator. The contact of the toluene regulator

¹ Designed by J. Wylie, B.A., Lecturer in Physics, Queen's University, Belfast.

is adjusted to cut off the large lamp when the temperature reaches the desired level, and throw in the small lamp which is outside the bath. The small lamp is useful for reading thermometer or other instruments in the bath.

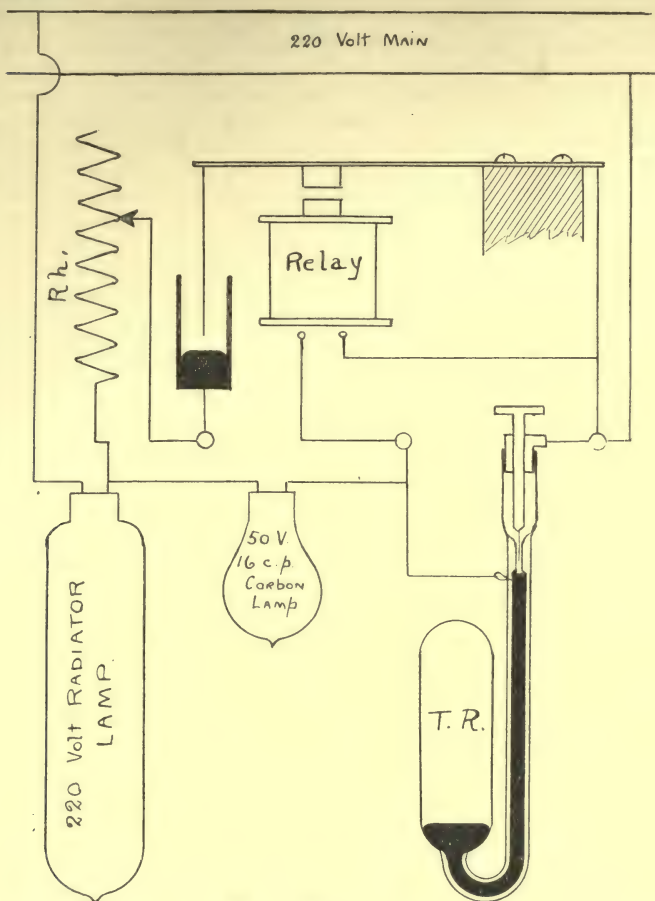


Fig. 4.

ROUTINE PROCEDURE IN THE ELECTROMETRIC DETERMINATION OF PH.

A free stream of pure hydrogen¹ is passed through the hydrogen electrode vessel for about half an hour, then after washing out the

¹ Details regarding platinisation of electrodes, preparation of hydrogen, calomel electrodes, etc., may be obtained from such books as Clark's "Determination of Hydrogen Ions," 1920.

vessel with the solution to be examined a fresh specimen of the latter is introduced under hydrogen pressure until the vessel is slightly more than half filled. If the Clark type of electrode be used (as shown in fig. 3), the saturation of the solution with hydrogen is effected by rocking the vessel up and down for a few minutes. The electrode vessel is now placed in such a position that the platinum plate just makes contact with the fluid, and connection is then made with the decinormal calomel electrode through the connecting tube containing 3.5 n. KCl. The accumulator circuit should have been closed for about ten minutes before connecting up the standard cell with the potentiometer. This having been done the rheostat in the accumulator circuit is adjusted until the galvanometer shows no deflection, so that the d.p. between the standard cell contacts is fixed and the potentiometer becomes a direct reading one. The .1 n. calomel electrode/hydrogen electrode system is now substituted for the cadmium cell, and the balance point of the former determined, in the first place, by moving the contact of the potential coils to the approximate probable voltage of the combination (fixing the e.m.f. to .1 volt), and, in the second place, moving the contact on the slide wire until slight movement to one or other side gives opposite deviation of the galvanometer coil. The e.m.f. is thus read to a fraction of a millivolt. The hydrogen electrode is again rocked until the readings become constant.

EXAMPLES.

(1) *A specimen of filtered gastric contents of well-marked acidity was introduced into the hydrogen electrode, and the e.m.f. of this electrode connected with the decinormal calomel one determined at 18° C. The final voltage was found to be .4484.*

$$\therefore \text{pH} = \frac{.4484 - .3377}{.0577} = 1.91$$

(2) *A specimen of the morning urine was treated in the same way, and the e.m.f. of the chain found to be .6313 volt = pH 5.08.*

(3) *Urine passed two hours later (after breakfast) gave .6892 volt, or pH 6.09.*

In the case of blood specimens special precautions require to be taken. In the first place, the carbonic acid tension in the blood should not be allowed to alter, and so the first blood sample in equilibrium with the gas in the electrode vessel should be replaced by a second one without displacing the gas mixture. In the second place, complete reduction of the blood pigment is necessary in cases where whole blood is under examination.

A comparison should be made between the results obtained by the indicator and the electrometric methods in the case of *standard* solutions.

The influence of the Hydrion Concentration on the Physical Properties of Protein Solutions.

Proteins and the amino-acids of which they are built up belong to the class of amphoteric electrolytes, that is to say, they can act either as acids or as bases, forming, in the first case, salts with strong bases, and, in the second, salts with strong acids. Thus the addition of such an amino-acid as phenyl-alanine to a standard acetic-acetate mixture of pH 4 will lower the cH, acting therefore as a base, while it will raise the cH in a similar mixture of pH 5, acting therefore as an acid. Midway between these values the addition of phenyl-alanine will scarcely alter the pH, and at the point of least disturbance the amino-acid is in the form of an internal salt, and will, in an electric field, show no preponderant tendency to move towards anode or cathode. This is the *isoelectric point*, and at this reaction protein solutions, as well as the amino-acids, exhibit certain important properties. These are mainly dependent upon the fact that at this reaction the proportion of the undissociated protein molecules to the total concentration, dissociated and undissociated, reaches its maximum. It is therefore at this point that the protein exhibits minimal stability and hence maximal precipitability. When such a solution is placed in an electrical field the transport of the protein shows no distinct unidirectional movement towards anode or cathode, while if the acidity be increased the migration becomes cathodic, and, if decreased, anodic.

The isoelectric points of certain proteins have been determined by Michaelis and others

	cH.
Serum Albumin (Native)	2×10^{-5}
Serum Globulin	0.6×10^{-5}
Caseinogen	3.6×10^{-5}
Oxyhæmoglobin	1.8×10^{-5}

The isoelectric point can be most readily arrived at in many cases by determining the reaction at which maximal precipitation occurs.

Dissolve some freshly precipitated caseinogen in decinormal sodium acetate solution. Place 10 c.c. samples of this mixture in a series of tubes and, by the addition of a gradually increasing quantity of N/100 and finally N/10 acetic acid, form a series of solutions of ascending cH from pH 6 to pH 3.5, noting the tube which shows optimal precipitation. With indicators determine the approximate reaction of this solution. The most distinct precipitate is found to be in the tube showing a pH of 4.8 approximately.

Experiments illustrating the influence of hydrion concentration on the action of enzymes, the viscosity of protein solutions, etc., can be readily carried out, and for work of this kind a water bath with accurate temperature control is requisite (fig. 4).

III.—Surface Tension

Various methods are employed for the determination of the surface tension of the body fluids. The methods most generally used in physiological work furnish only approximate values, but as very distinct alterations in surface tension are readily produced in body fluids, as, for example, by addition of bile salts, or by the action of lipase on simple glycerol esters, the accuracy of the methods is sufficient for such purposes. The most commonly employed method in biological work depends upon the estimation of the number of drops which fall from a horizontal circular plane surface in the discharge of a fixed volume of fluid, the standard for comparison being the number of drops from the same volume of water falling under exactly the same conditions. With a fall in surface tension of the solution, there is an increase in the number of drops from a given volume. The instrument commonly employed is the stalagmometer of Traube (fig. 5).

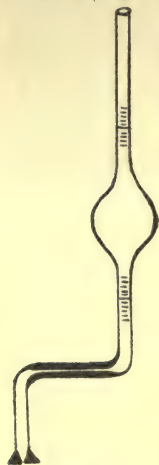


Fig. 5.

The calculation of the surface tension constant of a fluid is made as follows:—If d_w =drops of water, d_f =drops of the solution, s_w =sp. gr. of water (=1), s_f =sp. gr. of solution, a_w =surface tension constant of water (=7.45), and a_f =surface tension constant of the solution, then

$$a_f = 7.45 \frac{s_f \times d_w}{d_f}$$

The following solutions may be tested:—

1. Water.
2. Bile in water (1-1000 and 1-10,000).
3. Trace of bile salts in 10 c.c. water.

The water is first drawn up by suction (rubber ball) slightly over the upper mark above the bulb. It is then allowed to drop and the number of drops counted from the moment the upper mark is reached until the water falls to the lower mark. The same procedure is repeated with the solution, the number of drops being again counted. Repeat the process thrice and note whether the results are constant.

It is evident that a very slight addition of bile or bile salts to water distinctly increases the number of drops falling. Hay's sulphur suspension test is based upon the lowering of surface tension produced by the presence of bile salts in the solution.

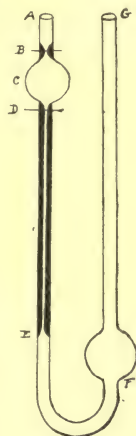


Fig. 6.

IV.—Viscosity

Determinations of "relative" viscosity are frequently of value in biological work as the internal friction of the animal fluids is largely determined by their colloidal content. The estimations may be conveniently carried out in the case of solutions free from suspended material by the use of such an instrument as Ostwald's viscosimeter (fig. 6).

A fixed volume of fluid, the amount varying with the capacity of the instrument, 5 to 10 c.c. is introduced into the wide limb (G), and suction is applied to the other end (A) until the solution has reached the mark (B) above the small bulb (C). With a stop-watch the time is then taken for the descent of the solution to the lower mark (D). This procedure is first carried out with water and then with the solution to be examined.

If t_0 = time of discharge of water of sp. gr. s_0 (=1) and friction coefficient n_0 (=1), then when t_1 , s_1 , and n_1 represent the corresponding values for the solution—

$$n_1 = n_0 \frac{s_1 t_1}{s_0 t_0} = \frac{s_1 t_1}{t_0}.$$

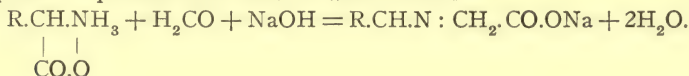
The viscosimeter should be placed in a glass tank filled with water kept at constant temperature. The following fluids may be examined:—water .9 per cent. NaCl, gum arabic saline, blood plasma, and bile.

CHAPTER XVIII

DETERMINATION OF AMINO-ACID-NITROGEN

I. DETERMINATION of amine-nitrogen by the formaldehyde titration method.

When a neutral solution of formaldehyde is added to a neutral solution of an amino acid, containing therefore an equal number of amine and carboxyl groups (pH 6.8--Sørensen), the mixture acquires an acid reaction owing to the conversion of the basic amine group ($R.NH_2$) into a neutral methylene group ($R.N:CH$) an equivalent amount of acid in the form of carboxyl ($R.CO.OH$) being set free (Schiff). The latter is then determined by titration with N/5 alkali. The following equation represents the reaction which occurs:—



(Neutral amino acid)

The reaction is a reversible one, the conversion into the methylene derivative being only complete in presence of excess of formaldehyde and of hydroxyl ions. The hydroxyl ions must be in sufficient concentration to give a deep red colour with phenolphthalein (pH 9--Sørensen). When dark coloured fluids have to be titrated thymolphthalein may be used instead of phenolphthalein. The colour change with the latter indicator occurs at a still higher hydroxyl ion concentration.

If considerable amounts of phosphates, or carbonates or both be present, these must be removed by precipitation with barium chloride and barium hydrate.

When the absolute amount of amino-acid-nitrogen requires to be determined, the method resolves itself into two parts. First the solution must be titrated with alkali or acid until it acquires the reaction pH 6.8, to ensure that the amine and carboxyl groups are present in equal numbers. Secondly, after the addition of neutral formaldehyde the solution must be titrated until it gives a deep red colour with phenolphthalein.

In studying the course of a proteolytic digestion the process may be greatly simplified, since it may be assumed that the quantity of fixed acid or alkali in the original solution remains constant, and that the

DETERMINATION OF AMINO-ACID-NITROGEN 285

only change which occurs during proteolysis consists in a new formation of equal numbers of amine and carboxyl groups. It must be remembered, however, that basic and acid ampholytes are also formed. The initial neutralisation of the solution to litmus paper may therefore be omitted (Sørensen).

1. Determination of the amount of amino-acid-nitrogen set free during a tryptic digestion.

REAGENTS REQUIRED.

(1) *Phenolphthalein solution*.—0.5 grm. phenolphthalein dissolved in 50 c.c. of alcohol + 50 c.c. water.

(2) *Formaldehyde solution (freshly prepared for each estimation)*.—Add 1 c.c. of the phenolphthalein solution to 50 c.c. of commercial formol (30 to 40 per cent.), and sufficient N/5 caustic soda (free from carbonate) or N/5 barium hydrate to give the solution a faint permanent red colour (formol solutions are always acid owing to the presence of formic acid).

(3) *Control solution*.—To 20 c.c. of distilled water (freed from CO_2 by boiling) add 10 c.c. of the formol solution and 5 c.c. N/5 barium hydrate or caustic soda, then add N/5 hydrochloric acid until the solution becomes faintly red (1st stage), again add one drop of N/5 alkali (a distinct red—2nd stage), and finally add another drop of N/5 alkali (a deep red colour—3rd stage).

Estimation.

Place 5 grms. of commercial "peptone" (caseinogen, gelatin or other protein may replace the peptone) in a 250 c.c. flask, add about 100 c.c. water, 10 c.c. of liquor pancreaticus or other enzyme preparation, and sufficient N/5 alkali to render the solution faintly alkaline to phenolphthalein. The amount of alkali required for this purpose may be ascertained by titrating a small portion of a control solution containing the same concentrations of peptone and the other constituents as the digest. Make up the solution to 250 c.c. with water and add a little toluene.

Determine the total nitrogen in 10 c.c. of the solution by Kjeldahl's method. With a pipette transfer 20 c.c. of the solution to a flask, add 10 c.c. of the formol solution (2), and titrate with N/5 alkali through stages 1 to 3, matching the final stage with the colour of the control solution. Note the number of c.c. of N/5 alkali used (say k). Place the digest in a stoppered flask in an incubator at 37° for 48 hours, then cool the digest, transfer 20 c.c. of it to a flask and titrate in the same way as before. Note the number of c.c. of N/5 alkali used (x). Since 1 c.c. of N/5 alkali is equivalent to $14/5$ or 2.8 milligrams of amino-acid-nitrogen, $(x - k) \times 2.8 =$ number of milligrams of amino acid and ammonia nitrogen in 20 c.c. of the digest set free during 48 hours' digestion of peptone by trypsin. Since the total quantity of nitrogen has also been determined, the percentage of

nitrogen, which has been converted into amino acids and ammonia, may be readily calculated. The digestion should then be continued for a week, and a third titration made, the amount of amino-acid- (and ammonia) nitrogen set free being calculated in the same way as before.

The foregoing method is sufficiently accurate for comparative estimations. A more accurate technique is described by Henriques and Gjaldbaek ("Zeitschr. f. physiol. Ch.," vol. lxxv. p. 63).

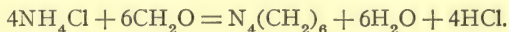
A peptic digest of gelatin, fibrin or other protein may be examined in a similar way, 150 c.c. N/10 hydrochloric acid being used as the medium, and the solution being made up to 250 c.c. as before. The period of digestion should be at least 7 days.

Sources of error.—The titration yields uncertain results with proline and tyrosine, the former giving too low and the latter too high titration values. Since these two sources of error partially balance one another, the error is not appreciable unless the percentage of either of these amino acids yielded by the protein is high.

The reaction of solutions of urea, and guanidine compounds such as creatine and arginine (the α -amino group being excepted in the latter case) is not affected by the addition of formol. Salts of the latter, *e.g.* the hydrochlorides may therefore be titrated as monobasic acids.

A more important source of error is the fact that the hydrion concentrations of the digests is not constant rising in the earlier stages of a tryptic digest, and falling again in the later stages (J. Christiansen).

INFLUENCE OF AMMONIUM SALTS.—Ammonium salts react with formaldehyde, yielding hexamethylenetetramine and an amount of acid equivalent to the ammonium originally present.



When ammonium salts and amino acids are both present, the formol titration yields lower values than the sum of the ammonia- and amino-acid-nitrogen (de Jager). In the presence of urea (which acts in some way that is not fully understood) the true value for the sum of ammonia and the amino-acid-nitrogen is found (de Jager). The quantity of ammonia formed during tryptic digestion does not greatly influence the titration values.

Highly coloured solutions require to be decolorised or matched with an artificially coloured control (*e.g.* one coloured by Bismarck brown). Sørensen has found the production of a precipitate of silver chloride in the solution acidified with hydrochloric acid the best method of decolorisation. The use of animal charcoal for decolorisation causes a loss of nitrogen, which however may be prevented by the addition of alcohol (Bang).

2. Determination of the amino acid and ammonia nitrogen in urine.

REAGENTS.—(1), (2), (3) as in 1.

(4) *Sensitive litmus paper*.—Dissolve 0.5 gm. of powdered azolitmin in 200 c.c. of water and 22.5 c.c. of N/10 caustic soda, filter, and mix with 50 c.c. of alcohol. Pass strips of ash free filter paper through this solution, and dry them suspended on threads for about an hour. The litmus paper should be tested against the following solutions, and should react as described below:—

3 c.c. $\frac{M}{15}$ Na_2HPO_4 , $2\text{H}_2\text{O}$ + 7 c.c. $\frac{M}{15}$ KH_2PO_4	faintly acid (= 6.47).
5 c.c. " " " + 5 c.c. " "	neutral (= 6.81).
7 c.c. " " " + 3 c.c. " "	faintly alkaline (= 7.17).

If the litmus paper do not react to the phosphate solutions in the way described, the quantity of N/10 alkali must be altered as required.

When, as in urine, the absolute amounts of amino acid and ammonia nitrogen have to be determined, the titration must start from a definite hydron concentration as zero. The investigations of Sørensen and Henriques have proved that the most accurate results are obtained when a faintly acid reaction (pH 6.8) is taken as the starting-point. At this reaction the number of amine groups is equal to that of carboxyl groups.

METHOD OF ESTIMATION.

Measure 50 c.c. of urine with a pipette into a 100 c.c. flask, and add 1 c.c. of phenolphthalein solution, and 2 grms. of barium chloride. Shake until the barium chloride has dissolved, then add drop by drop a saturated solution of barium hydrate until the urine just becomes permanently red, then add an additional 5 c.c. of the barium hydrate solution, fill up to the mark with water, shake thoroughly, allow to stand for 15 minutes, and finally filter through dry filter paper. The urine, if highly coloured, may be decolorised without loss of nitrogen by adding 20 c.c. 95 per cent. alcohol before making up to 100 c.c., then adding about a teaspoonful of animal charcoal, shaking up thoroughly, and filtering as before.

Neutralise 40 c.c. of the filtrate (= 20 c.c. of urine) by adding N/5 HCl until the red colour is discharged (if charcoal have been used for decolorisation it will be necessary first to add a few drops of the phenolphthalein solution), then continue the addition of the N/5 acid until a drop of the fluid gives a neutral reaction with the litmus paper. A drop of the second phosphate mixture, placed on an adjacent portion of the litmus paper, may be used as a standard.

Add 20 c.c. of the neutral formol solution and titrate with N/5 barium hydrate or caustic soda through the first and second stages until the third stage (deep red of same tint as control) is reached. The number of c.c. of N/5 alkali used $\times 2.8$ gives the sum of the ammonia-N and amino-acid-N expressed in milligrams present in 20 c.c. of urine. The ammonia-N is determined by the method given in the chapter on the quantitative examination of urine, and the

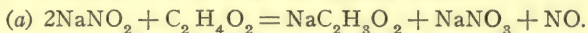
amino-acid-N may be obtained by deducting the ammonia-N thus found from the sum of the two forms of nitrogen.

The amount of urea present in the urine is sufficient to enable one to obtain sufficiently accurate results for the sum of ammonia and amino-acid-nitrogen by the direct method just described. A more accurate (but also more complicated) method involving the preliminary removal of preformed ammonia by distilling the urine *in vacuo* with barium hydrate has been devised by Henriques and Sørensen. The same observers have also modified the method so as to include in the result the glycine present as hippuric acid together with the nitrogen of polypeptides ("Zeitschr. f. physiol. Chemie," vol. lxiv. (1910), pp. 120-143).

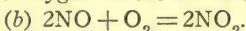
II. Estimation of aliphatic amino groups (Van Slyke's method).

PRINCIPLE OF THE METHOD.—This method is based on the measurement of the nitrogen evolved when aliphatic amino groups are converted into hydroxy groups by the action of nitrous acid (see p. 143). Ammonium salts and urea (and other amides) are decomposed in a similar way, but react much more slowly. Half of the nitrogen evolved is derived from the amino groups, and half from the nitrous acid. The amino-nitrogen therefore represents only half that measured. This fact is allowed for in the table given by Van Slyke (see p. 409).¹

The excess of nitrous acid is decomposed, thus—



Part of the nitric oxide remains unchanged, and part is oxidised to nitrogen peroxide by the oxygen of the air in the apparatus.



The air is first displaced from the apparatus by NO. Finally, after deamination of the amino acid, the excess of NO is oxidised and absorbed by alkaline potassium permanganate, being converted into potassium nitrate, and the residual nitrogen measured.

REAGENTS REQUIRED.

(1) 30 grms. sodium nitrite dissolved in 100 c.c. water. (2) 50 grms. potassium permanganate, and 25 grms. KOH or NaOH dissolved in water and made up to 1 litre. (3) Glacial acetic acid. (4) N/10 solution of an amino acid, *e.g.* 0.75 grm. of glycine dissolved in water and made up to 100 c.c.

METHOD.

1. Preliminary procedure.—Fill one bulb of the Hempel pipette (H) with (2). Fill the reservoir K with water, and by raising it displace the air in F, turning stopcocks (f) and (c) so that the air passes out through (c). Turn (f) so that the burette (F) communi-

¹ Although the table given on p. 409 is not so convenient as that of Van Slyke, it has the advantage of being applicable to the measurement of other gasses than nitrogen.

cates with the capillary tube leading to the Hempel pipette (H), and lower (K) until the alkaline potassium permanganate fills the capillary

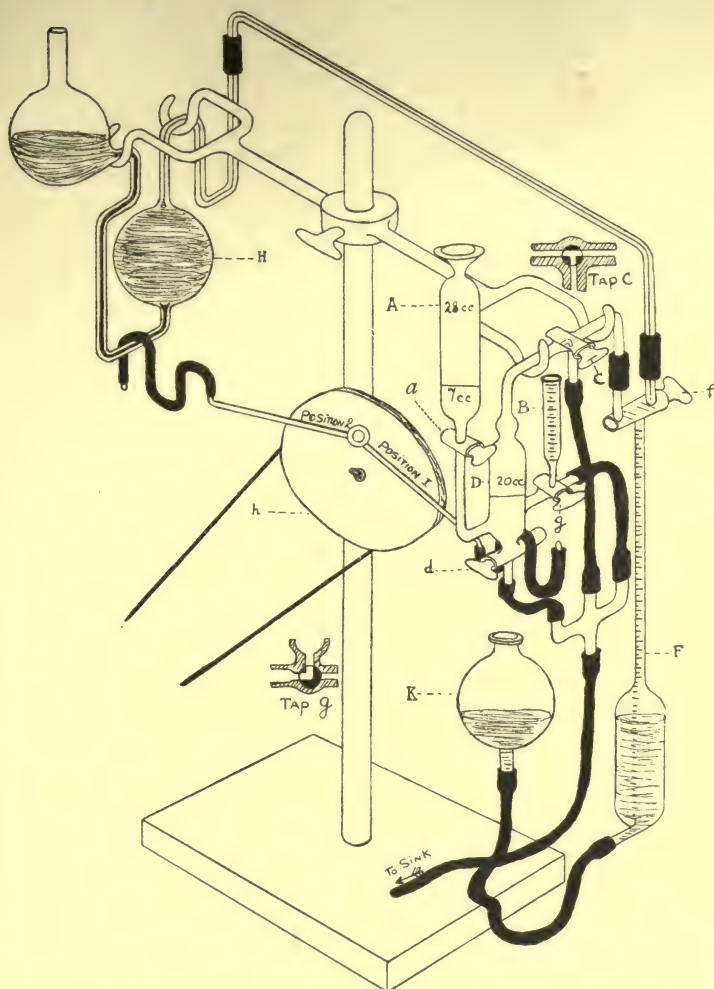


Fig. 7. .

tube and bore of stopcock (f). Finally, turn (f) so as to cut off H and connecting capillary tube from F. Raise (K) and turn (f) and (c) so that the air passes out through c; fill F and connecting tube up to (c) with water, and turn (c) so that F is cut off from

communication with the air. The parts of the apparatus mentioned are now filled with fluid.

2. Displacement of air by nitric oxide.—Place 7 c.c. of glacial acetic acid (sufficient to fill one-fifth of total capacity of A). Turn (c) so as to allow the air to escape from D to the exterior, and run the acid into D. Run in sodium nitrite solution from A to D until D is filled, and sufficient excess of sodium nitrite to rise a little above (a). Close (c), and (a) being open shake D for a few seconds. Let out the nitric oxide, which is evolved, at (c), and repeat the shaking. NO is again evolved and let out at (c). The air in D and connecting tube up to (c) is now replaced by NO. Close (c), open (a), connect D with the motor, and shake until all but 20 c.c. of the solution have been displaced by NO and driven back into A. Close (a) and turn (c) and (f) so that D communicates with F.

3. Decomposition of the amino-substance.—Measure 10 c.c. of the amino-acid solution into B. Lower the reservoir K so as to establish a pressure slightly lower than atmospheric in F and D, and run in the amino-acid solution into D, washing out B with a little water. Connect D with motor and shake for 3 to 5 minutes. The gases evolved pass into burette F. Finally, drive all the gas in D into F by opening (a) and filling D with solution from A up to (f), and close (f).

4. Absorption of nitric oxide and measurement of nitrogen.—Turn (f) so as to connect F with the Hempel pipette H. Drive the gas into the pipette by raising K. Connect H with the motor, and shake for 1 to 2 minutes. Lower K so as to bring back the gas into F, and measure the volume of gas, bringing the water in K and F to the same level. Drive over the gas again into H, repeat the shaking, bring back the gas into F, and again measure. If the volume be unaltered, note the temperature and barometric pressure.

Ascertain from Van Slyke's Table the weight in milligrams of amino-acid nitrogen corresponding to 1 c.c. of nitrogen at the prevailing temperature and pressure. This value multiplied by the number of c.c. of gas measured gives the weight in milligrams of amino-nitrogen present in the 10 c.c. of the solution of an amino-acid analysed.

The measuring burette (F) with bulb has a total capacity of 150 c.c. The upper narrow portion of the burette has a capacity of 40 c.c. divided into 1 c.c. and 0.1 c.c.

ADDITIONAL NOTES.

(1) The potassium permanganate solution in H is sufficient for 10 to 12 estimations.

(2) Since sodium nitrite frequently contains substances which yield nitrogen, a blank experiment should be carried out, replacing the 10 c.c. of amino acid solution with 10 c.c. of water. If the volume of nitrogen evolved exceed 0.3 to 0.4 c.c., the nitrite should be rejected. If less than 0.3 to 0.4, the value obtained should be deducted from the volume of nitrogen yielded by the amino acid.

(3) At 20° the α -amino groups react quantitatively with nitrous acid in 5 minutes. The ϵ -amino group in lysine requires 30 minutes for complete reaction. Ammonia reacts completely in 1½ to 2 hours, and urea in about 8 hours (50 per cent. in one hour). The guanidine group (present in creatine and arginine) does not react. Consequently only one of the four nitrogen atoms in arginine reacts, namely, that in the α position. The nitrogen in the indole (*e.g.* of tryptophane), pyrrolidine (*e.g.* of proline and hydroxy proline), and iminazole (*e.g.* histidine) rings also does not react. Hence tryptophane yields half its nitrogen, histidine one-third of its nitrogen, and proline no nitrogen on treatment with nitrous acid. The α -amino group of asparagine reacts quantitatively in 5 minutes, the amide group only after some hours. Glycine yields more gas not absorbable by alkaline potassium permanganate than the theoretical amount (103 per cent.). Cystine yields 7 per cent. more gas than the theoretical volume of nitrogen.

In polypeptides only the free amino groups react. Egg albumin yields 2.98 per cent. of its nitrogen, primary proteoses about 6.3 per cent. secondary proteoses about 10 to 14 per cent.

(4) If the solution to be analysed is likely to froth violently, a little caprylic alcohol should be run in from B, before adding the amino-acid solution. B is then washed out with ethyl alcohol and dried with filter paper.

Micro-Apparatus.—Van Slyke has also devised a smaller form of apparatus suited for the analysis of small quantities of material.

Applications of D. Van Slyke's Method.—The chief applications of the method are the following:—

(1) Identification of amino acids.

(2) Determination of the "distribution of nitrogen," or forms in which nitrogen is present in the products of hydrolysis of proteins.

(3) Determination of amino acids in urine, blood, and other fluids.

For these, the original papers may be consulted. ("J. Biol. Chem.," vol. xii. 275; vol. xvi. 121; vol. xxiii. 408.)

CHAPTER XIX

URINE

URINE

1. Quantity.

The urine should be collected in an absolutely clean glass vessel containing a small quantity of toluene. In daily analyses the collection should always begin with the bladder emptied at a fixed hour in the morning, and the urine collected until same hour on the following day, the urine passed then being added to that passed during the rest of the twenty-four hours. It is, as a rule, sufficient to measure the volume of the urine passed during the twenty-four hours in a measured cylinder; but if a series of analyses is being made of various urinary constituents, it is advisable subsequently to pour the urine into a measured stoppered flask of rather larger capacity than is required, and then fill up to the mark on the flask with distilled water, or, if necessary, with a suitable solvent for any deposit in the urine. When this is done, carefully measured portions may be taken for the various analyses, the measurements being always taken in standard measured glass flasks which have been carefully cleaned and dried. Sets of such flasks should be kept in stock, any errors in their capacity (as stated on flask) being noted, and the necessary correction made. The measurement should always be taken at one fixed temperature (15° to 20°).

2. Specific gravity is usually taken with—

(a) The URINOMETER, a method which is sufficiently exact if the estimations are only being made in a fluid the character of which does not alter much from day to day. It

is very advisable to have at least two sets of urinometers, one graduated from 1000 to 1020, and the other from 1020 to 1040. It is also advisable to take the density at the temperature marked on the instrument (say 15°), rather than make the correction (addition of .001° for every 3° above 15°, and subtraction of same for every 3° below that temperature). Any deposit that clears up easily on heating (*e.g.* urates) should be dissolved, the temperature correction then requiring to be made.

In taking the density, see that the surface is free from froth and impurities (remove with close filter paper), then carefully immerse bulb of urinometer in urine, and allow it to float free in the centre of the cylinder. It must not come into contact with any part of the vessel.

Take the reading with the cylinder on a flat surface, and, with the eye level with the lower border of the fluid meniscus, then note where this cuts the scale of the instrument.

[(b) With PYKNOMETER.]

The weight of a given volume of urine is compared with that of the same volume of distilled water at a fixed temperature (15°), the latter being taken as unity.

The best vessel to use for this purpose is a small stoppered flask, which does not require to be absolutely filled, but has a constriction on the neck with a mark upon it. This saves introduction of errors caused by evaporation through bored stoppers, overflowing of urine during the filling process, and the consequent necessity for cleaning and drying the surface.

The specific gravity of a fluid (urine) can be calculated when the weight of the empty flask is known, also its weight when filled with distilled water at a given temperature, and again when filled with the fluid.

a = weight of empty flask; b = flask + water at 15°;
 c = flask + urine at 15°.

$$\frac{c-a}{b-a} = \text{specific gravity of urine.}$$

Take small flask of above-mentioned type, wash

thoroughly with water, alcohol, and ether, dry and weigh. Then fill with distilled water up to mark on neck, and weigh. Empty flask, wash out with alcohol and ether, dry, and then fill with urine in same manner, and weigh. The temperature must be the same throughout (15°). The calculation of the specific gravity is made in the way above described.

3. Total Solids.

The method usually employed of evaporating down a fixed volume of urine on the water bath and drying in exsiccator to constant weight (?) is practically valueless, as constant decomposition of urea takes place during the evaporation, and it is impossible to arrive at constant weight without decomposition, unless precautions are adopted which render the method much more complicated than the value of the results obtained merits. The incineration of the solids is only necessary when one suspects the presence of an inorganic constituent which cannot be detected in the fluid urine by the ordinary tests. One can arrive at a rough knowledge in many cases of the amount of solids in the urine, from the specific gravity. Christison's formula, namely, the multiplication of the last two figures in the specific gravity (stated in terms of 1000) by 2.33, gives the number of grm. solids in 1000 c.c. urine. The method can of course only give an indication of the amount of solids.

4. (1) Titratable acidity ; (2) determination of ammonia- and amino-acid-nitrogen ; and (3) phosphate-ratio.

(1) No method is very satisfactory for urine. A sufficiently accurate result can, however, be obtained in the following way :—

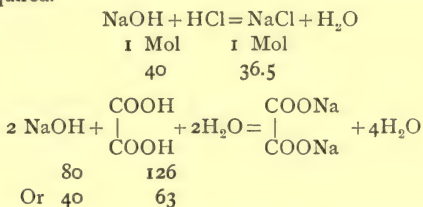
Fill a burette (Schellbach's) with decinormal NaOH, seeing that all air bubbles have been driven out from the discharging point. Take the reading of the level of the fluid, which is easily done, as the point is exactly shown where the constriction of the black or blue line appears. From another similar burette, or a pipette, run 20 c.c. of

the mixed urine of twenty-four hours into a small flask, then add 80 c.c. of distilled water (varying with density of colour of urine). Take the same quantity of urine, diluted to same extent, in a similar flask, to act as a control for the end reaction.

To each one add a few drops of a 1 per cent. alcoholic solution of phenolphthalein. Now, into one of these gradually run in the decinormal NaOH, shaking after each addition, until a red tint appears, which remains for about a couple of minutes. The tint of the control urine always aids in noting the point of the end reaction. It is also advisable to place both flasks on a large sheet of white paper.

$$\text{Each c.c. of the } \frac{N}{10} \text{ NaOH} = 1 \text{ c.c. of any } \frac{N}{10} \text{ acid.}^1$$

¹ By a normal solution is meant one in which the hydrogen equivalent of the substance in grammes is dissolved in a litre of water. This, for a monobasic acid, is just its molecular weight in grammes; for a dibasic, half; and for a tribasic, one-third, of its molecular weight in grammes. As examples of mono- and dibasic acids respectively, hydrochloric and oxalic acids may be taken. To neutralise one molecule of NaOH, one of HCl or half a molecule of oxalic acid is required.



The 2 mol of water of crystallisation have to be added to the molecular weight of oxalic acid.

That is to say, if 40 grms. NaOH are dissolved in a litre of water, the solution will be neutralised by addition of 36.5 grms. HCl or 63 grms. C₂H₂O₄ dissolved in a litre of water. Each c.c. of the alkaline solution will equal each c.c. of the acid ones; and each c.c. of the latter contains the same amount of acid in milligrammes that the litre contains in grammes.

The above-mentioned solutions are "normal" ones, but frequently weaker solutions, e.g. decinormal, one-fifth or half-normal, are employed.

Thus every c.c. of $\frac{N}{10}$ oxalic contains 6.3 mgrms. of that acid; and every c.c. of $\frac{N}{5}$ HCl = 7.3 mgrms. of that acid.

The results may be stated in terms of any acid.¹ If in terms of HCl, then as each c.c. of $\frac{N}{10}$ HCl contains $\frac{36.5}{10}$ mgrms. HCl, the degree of acidity of the 20 c.c. (in mgrms. HCl) will equal the number of c.c. of decinormal alkali used, multiplied by 3.65. And, knowing the total twenty-four hours' urine, the total acidity can be at once calculated.

(2) **Ronchèse and Malfatti's Method for the Estimation of "Ammonia"** (ammonia and amino acids).

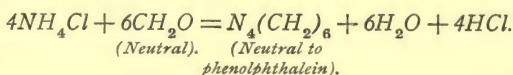
REAGENT REQUIRED.

Neutral solution of formaldehyde.

Dilute 10 c.c. of formol with 20 c.c. of water, add 4 drops of 0.5 per cent. phenolphthalein, and sufficient $\frac{N}{5}$ NaOH to give the solution a faint permanent red colour.

Add the foregoing reagent to the urine previously neutralised for the determination of acidity. Note that the red colour of the urine is discharged. Then add from a burette an accurately measured quantity of N/10 caustic soda until a permanent red colour is obtained. The number of c.c. of N/10 alkali used multiplied by 1.4 gives the quantity of ammonia-nitrogen expressed in mgrms. in 20 c.c. of urine.

The method is based on the following reaction, which is more fully explained on page 286:—



This equation shows that the amount of acid set free by the addition of neutral formaldehyde is equivalent to the quantity of ammonia.

This method only gives approximate results, and the amino acids of the urine are included in the determination. The amounts of the latter are normally very small. In clinical work the method yields valuable comparative results, when variations in the ammonia content of urine from the same individual are determined from day to day.

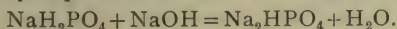
Folin has found that the end point in this titration is better defined if the calcium be precipitated by the addition of a neutralised saturated

¹ The acid output in the urine is now usually stated as the number of c.c. N/10 acid equivalent to the acid present in the total urine per day. The acid equivalent of the ammonia (determined by formol titration or preferably by the Krüger-Reich method) present in the urine should be added to this value in order to obtain the total output of acid in the urine.

solution of potassium oxalate (5 c.c. to 20 c.c. of urine). The further procedure is the same as that already described.

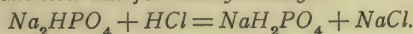
The method described in (1) gives no direct information with regard to the "real reaction or hydron concentration" of the urine.¹

(3) **Determination of the "phosphate-ratio."**—The titration value obtained by method (1) gives us the amount of alkali required to convert all the monobasic phosphate of the urine into dibasic phosphate, thus :—



When this conversion is complete, the slightest excess of alkali gives a red colour with phenolphthalein. The titratable acidity is therefore proportional to the quantity of monobasic phosphate in the urine. Let x be the number of c.c. of alkali required.

Add 4 drops of methyl-orange solution to the urine already neutralised to phenolphthalein, and titrate with N/10 hydrochloric acid to the transition tint for methyl-orange



When all the dibasic phosphate has been converted into monobasic phosphate, the slightest excess of acid gives a red colour with methyl-orange. Let y be the number of c.c. of N/10 acid required, then $y-x$ is the quantity of N/10 acid required to convert all the M_2HPO_4 of the original sample of urine into MH_2PO_4 , and is therefore proportional to the quantity of dibasic phosphate in the original urine.

Hence, by combining the results of the two titrations one obtains the ratio of monobasic to dibasic phosphate $\frac{\text{MH}_2\text{PO}_4}{\text{M}_2\text{HPO}_4} = \frac{x}{y-x}$.

The value y measures the capacity of the urine for neutralising alkali, or measures the range of pH between NaH_2PO_4 and Na_2HPO_4 , and most writers therefore prefer to state the ratio of monobasic and dibasic phosphates in terms of y taken as unity, 10 or 100.

CALCULATION.

Let $\frac{x}{y-x} = \frac{m}{n}$, and let $\frac{p}{q}$ = the same ratio, each term of the ratio being expressed as a fraction of y or $m+n$ taken as 10. Then $p = \frac{10m}{m+n}$ and $q = \frac{10n}{m+n}$.

EXAMPLE.

In this case, 10 c.c. of urine were titrated. x was found to be 6.2 c.c., and $y-x$ to be 2.7 c.c. Hence $p = \frac{62}{8.9} = 6.97$, $q = \frac{27}{8.9} = 3.03$, and $\frac{p}{q} = \frac{6.97}{3.03} = \frac{\text{MH}_2\text{PO}_4}{\text{M}_2\text{HPO}_4}$, or stated as percentages, y being taken as 100— $\frac{69.7}{30.3}$.

¹ See pages 264-280 for methods of determining "Reaction."

The following experiments may be carried out:—

Determine (1) the total volume and phosphate-ratio of the night's urine, (2) the corresponding values one hour after rising, no food being taken, and (3) the values one and two hours later, breakfast being included in the first period. If possible, carry out similar estimations with the urine of a case of acidosis of diabetic or other origin.

The significance of this ratio has been frequently referred to. The following papers may be consulted for further information and references to recent literature (Leathes, "British Medical Journal," 1919, vol. ii. p. 165, and Calvert, Mayrs, and Milroy, "Journal of Pathology," vol. xxiv. pp. 91 to 116).

Alkali Tolerance.

METHOD.

After emptying the bladder, take 2 grms. of sodium bicarbonate dissolved in 100 c.c. of water. Determine the phosphate ratio after an hour. If still fairly high take another dose of 2 grms. of sodium bicarbonate and determine the ratio at the end of another hour. As a rule 0.5 grms. sodium bicarbonate per kilo of body weight will produce urine alkaline to phenolphthalein in a normal individual. The effect of administration of alkali should always be controlled by determination of the phosphate-ratio, and not carried too far to avoid the risk of producing "alkalosis." If possible, similar experiments should be carried out with the urine of a case of acidosis (Sellards, Palmer, Henderson, and Van Slyke. The reader is referred to Palmer and Van Slyke, "Journal of Biol. Chem.," vol. xxxii. p. 499).

5. Chlorides.

Two methods are employed, in both of which AgNO_3 is used to precipitate the chlorides.

(a) VOLHARD'S METHOD.

This depends upon the complete precipitation of the chlorides in nitric acid solution by AgNO_3 , and the estimation of the excess of AgNO_3 present after all the chlorine has been precipitated.

This excess is estimated by adding a standard solution of ammonium sulphocyanate,¹ which precipitates the soluble silver salt as insoluble silver sulphocyanate, the end point being noted by adding a ferric salt to the solution. This

¹ See note on p. 301.

salt reacts with ammonium sulphocyanate to give ferric sulphocyanate (red), but does not react with silver sulphocyanate. The silver nitrate is made up of such a strength that 1 c.c. will completely precipitate the chlorine of 10 mgrms. NaCl.¹

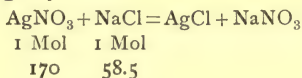
There are two ways in which the estimation can be carried out:—

1. Usual Method.

Run 10 c.c. urine into 100 c.c. measured flask, then add about 50 c.c. distilled water and 4 c.c. of pure 30 per cent. HNO_3 (free from nitrous acid and chloride). Now, from a burette run in 10 to 20 c.c. of the standard AgNO_3 , taking care that more is added than is required to precipitate all the chloride. The supernatant fluid may be tested to see that all the chloride is down, by adding 1 -- 2 c.c. more of the standard AgNO_3 . Now, fill up to the 100 c.c. mark with distilled water, shake thoroughly and filter through a dry folded filter paper, previously washed chlorine free. The filtrate should be collected in a dry flask, 50 c.c. transferred with a pipette to a second flask, and the excess of the AgNO_3 added is then to be estimated in this portion of the total.

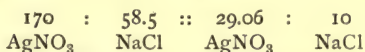
This is done as follows:—Add 5 c.c. of a 3 per cent. solution of ammonia iron alum (chlorine free) to 50 c.c. of the filtrate. Now run in from another burette

¹ Standard AgNO_3 (1 c.c. = 10 mgrms. NaCl).—The strength can easily be calculated in the following way:—



That is to say, 170 parts AgNO_3 will precipitate the chloride of 58.5 part NaCl. The strength of AgNO_3 requisite in order that every c.c. should equal 10 mgrms. NaCl, would be obtained by dissolving in 1 c.c. the equivalent weight of AgNO_3 for that quantity of NaCl (10 mgrms.).

That is to say—



Or, if 29.06 grms. of pure fused AgNO_3 be dissolved in 1 litre of water, every c.c. of this solution will be equivalent to 10 mgrms. NaCl.

standard ammonium sulphocyanate solution (1 c.c. of this = 1 c.c. of the AgNO_3), until a permanent red tint of the iron sulphocyanate appears. The number of c.c. of the NH_4CNS used gives the number of c.c. AgNO_3 in excess over that required to precipitate all the chlorides. This does not represent the whole of the excess of AgNO_3 , but 0.5, as only 50 c.c. of the original 100 were used.

An example will make this clear.

Example.

10 c.c. urine taken (total twenty-four hours' excretion 1500 c.c.), made up to 100 c.c. as described, 20 c.c. standard AgNO_3 being taken. Of the total 100 c.c. 50 c.c. were taken, and in this an excess of 6 c.c. AgNO_3 was found (that is, 6 c.c. NH_4CNS were required). That is, 12 c.c. standard AgNO_3 were present in the 100 c.c., after all the chlorides were precipitated.

20 c.c. were originally added ;

$\therefore 20 - 12 = 8$ c.c. AgNO_3 were required to exactly precipitate all the chlorides in 10 c.c. urine.

(The standard AgNO_3 solution has been made of such a strength that each c.c. = 10 mgrms. NaCl .)

$\therefore 8 \times 10 = 80$ mgrms. NaCl in 10 c.c. urine ; and

$80 \times 150 = 12$ grms. in 1500 c.c. urine.

[2. Rapid Method.]

This method is not so reliable as that just described.

As a rule the AgCl is removed by filtration before estimating the excess of AgNO_3 . This is done in order to prevent any formation of silver sulphocyanate from the action of ammonium sulphocyanate on silver chloride. This interchange, however, takes place so very slowly that it is not necessary, with moderately rapid working, to remove the AgCl before estimating the AgNO_3 excess.

Take 10 c.c. urine, add about 90 c.c. distilled water, 5 c.c. of the ammonia iron alum solution, and sufficient HNO_3 just to decolorise the ferric salt. Run in excess of standard AgNO_3 (say 20 c.c.). Now, quickly run in the sulphocyanate solution, shaking continuously, until the light-brown tint of the iron sulphocyanate becomes permanent in the supernatant liquid (should remain five minutes at least).

The amount of NH_4CNS^1 gives at once the excess of AgNO_3 used beyond the quantity required to precipitate all the chlorides in 10 c.c. urine. Knowing the original number of c.c. AgNO_3 taken, and the excess, the difference will give the number used up, and each c.c. of this = 10 mgrms. NaCl .

(b) MOHR'S METHOD.

The principle of this method is the precipitation of all the chlorides by AgNO_3 (standard solution) in neutral solution. The end point is detected by adding a solution of neutral potassium chromate to the diluted urine prior to titration. Whenever the AgNO_3 has precipitated all the chloride, and a small excess of the soluble silver salt appears in the fluid, the alkaline chromate reacts with the latter, and a faint red tint of a trace of silver chromate appears.

The slight acidity of the urine does not interfere with the end reaction.

This method gives too high results for the urine, because other substances which are present, such as purine bodies, sulphocyanates, etc., combine with the silver before the chromic acid does, and therefore a correction requires to be made.

Example.

Take 10 c.c. of the twenty-four hours' urine in a beaker, dilute with about nine times its volume of water, neutralise with Na_2CO_3 solution,² and add five or six drops of a cold saturated solution of potassium chromate. Gradually run in from a burette standard AgNO_3 solution (1 c.c. = 10 mgrms. NaCl), stirring constantly with a glass rod, and continue doing so until the permanent faint red colour of the silver chromate appears.

From the number of c.c. AgNO_3 used, deduct 1 c.c.

¹ *Standard NH_4CNS .*—Dissolve 13 grms. pure NH_4CNS in 1 litre of water. Run in this solution from a burette into 20 c.c. of the standard AgNO_3 solution, to which 5 c.c. of the ferric salt solution and 4 c.c. of 30 per cent. HNO_3 have been added. Note the point when end reaction is given. Knowing the number of c.c. NH_4CNS required for 20 c.c. standard AgNO_3 , it is easy to dilute until 1 c.c. of the former equals 1 c.c. of the latter.

² In the case of urine this neutralisation is not absolutely necessary.

(the correction referred to), and on multiplying the remainder by 10, the number of milligrammes NaCl in 10 c.c. urine is given, and, knowing this, the total twenty-four hours' chloride excretion can be calculated; e.g. if 8 c.c. AgNO_3 are required for 10 c.c. urine, the total twenty-four hours' quantity being 1500 c.c.: then—

C.c. AgNO_3 .	Mgrms. NaCl.	Urine.
------------------------	-----------------	--------

$(8-1) \times 10 \times 150 = 10.5$ grms. NaCl in 24 hours.

Bang and Larsson's Modification.

Add about 1.5 gm. of animal charcoal (pure for analysis) to 20 c.c. of urine, shake vigorously, allow the mixture to stand for at least five minutes, then filter through a dry filter paper into a dry flask. 10 c.c. of the colourless filtrate are titrated with the standard silver nitrate (or with $N/10 \text{ AgNO}_3$) in the way already described. The number of c.c. of standard silver nitrate solution used $\times 10$ gives the quantity of sodium chloride in mgrms. present in 10 c.c. of urine. No deduction is necessary, since the substances, which interfere with the accuracy of the original method, are absorbed along with the urinary pigments by the charcoal. If $N/10$ silver nitrate be used, multiply by 5.85 instead of 10.

6. Phosphates.

TOTAL INORGANIC PHOSPHATES.

The method that is most commonly employed depends upon the precipitation of all the urinary phosphates by a standard solution of uranium acetate (or, better, uranium nitrate in the presence of sodium acetate and acetic acid), and the detection of the point when this is complete and a trace of excess of soluble uranium salt appears.

This end point may be shown by potassium ferrocyanide, which gives a brown precipitate with the soluble uranium salt; or by cochineal tincture, which becomes green in colour at the same point.

The uranium nitrate solution is so made that every c.c. = 5 mgrms. P_2O_5 .

This standardisation is carried out by titrating the uranium solution against a disodic phosphate solution, containing about the same amount of P_2O_5 as average normal urine. A

solution of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, containing 0.2 grm. P_2O_5 in 100 c.c., is prepared.

As this cannot satisfactorily be done by simply weighing the theoretical amount of the salt (10.094 grms. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and dissolving it in a litre of water (owing to the efflorescence of the crystals), it is usually carried out in the following way:—

Weigh about 12 grms. of the salt. Dissolve in a litre of water, and of this take 50 c.c. Evaporate this to dryness, incinerate and weigh as pyrophosphate. 50 c.c. of a 1.0094 per cent. solution of Na_2HPO_4 should furnish .1875 grm. of the pyrophosphate. From the amount obtained in the specimen which has been incinerated, one can calculate the amount of the original disodic phosphate in the 50 c.c., and therefore can dilute the solution, so that 1.0094 grm. are present in 100 c.c.

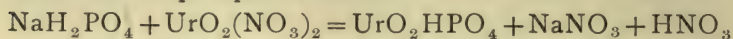
The uranium solution is made in the following way:—

35.461 grms. uranium nitrate, $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, are dissolved in rather less than a litre of water, and the solution is then standardised against the Na_2HPO_4 solution (containing .2 per cent. P_2O_5).

Take 50 c.c. of the Na_2HPO_4 solution in a porcelain basin, add 5 c.c. of the acetic acid sodium acetate solution (100 grms. sodium acetate, 30 grms. acetic acid made up to 1 litre with water), and a few drops of tincture of cochineal. Heat to boiling point, and add uranium solution until the mixture acquires a permanently faint green tint. As a control, take a drop out from the boiling solution, touch a drop of potassium ferrocyanide on a porcelain plate, and note if the brown end reaction be given; if not, add an extra drop or two until it is distinct. The solution should be of such a strength that 20 c.c. should be equivalent to 50 c.c. of the Na_2HPO_4 solution, which equals .1 grm. P_2O_5 . Supposing 18 c.c. were sufficient to precipitate the P_2O_5 of the 50 c.c., then 2 c.c. distilled water would require to be added to every 18 c.c., and the titration again carried out until 20 c.c. were equal to the 50 c.c. of the Na_2HPO_4 solution.

Then each c.c. of the uranium solution would be equivalent to 5 mgrms. P_2O_5 .

It is necessary to add the sodium acetate to neutralise the HNO_3 , which is set free when uranium nitrate reacts with a mono-alkaline phosphate: thus—



This free HNO_3 would prevent the precipitation becoming complete by breaking up the uranium phosphate. The acetic acid is employed in order to convert all the phosphates present in the urine into the mono-alkaline. It also, of course, prevents any earthy phosphates remaining undissolved, or a precipitate of uranium hydrate appearing, such as would occur were the solution alkaline.

Example.

Take 50 c.c. urine in an Erlenmeyer flask, add 5 c.c. of the acetate solution and a few drops of cochineal tincture. Bring to boiling point, and then run in, gradually, the uranium nitrate solution, until the green colour becomes permanent. It is advisable at this stage to take a drop of the supernatant liquid and bring it into contact with one of potassium ferrocyanide, in order to see whether the two indicators show the same end point. This is absolutely necessary if the urine be deeply coloured.

Note the number of c.c. uranium solution used; each c.c. = 5 mgrms. P_2O_5 . Say 20 c.c. used up = 100 mgrms. P_2O_5 in 50 c.c. urine, or 3 grms. P_2O_5 in twenty-four hours, if 1500 c.c. passed.

The phosphates of the urine may also be determined by Neumann's method. If the urine be first oxidised with sulphuric and nitric acids, the result obtained gives the total phosphorus—both inorganic and organic—of the urine. Neumann's method is more accurate than the uranium method, but not so suitable for class work.

7. Sulphates.

A. GRAVIMETRIC METHOD (Folin).

REAGENTS REQUIRED.

(1) Dilute hydrochloric acid (1 volume of concentrated acid diluted with 4 volumes water).

- (2) Concentrated hydrochloric acid.
Both must be free from sulphates.
(3) 5 per cent. barium chloride.

APPARATUS REQUIRED.

- (1) 25 and 50 c.c. pipettes ; (2) Erlenmeyer flask (200 to 250 c.c.) ;
(3) Gooch crucible.

Note.—PREPARATION OF ASBESTOS MAT.

Suspend a few grams of asbestos fibres in 300 c.c. of 5 per cent.

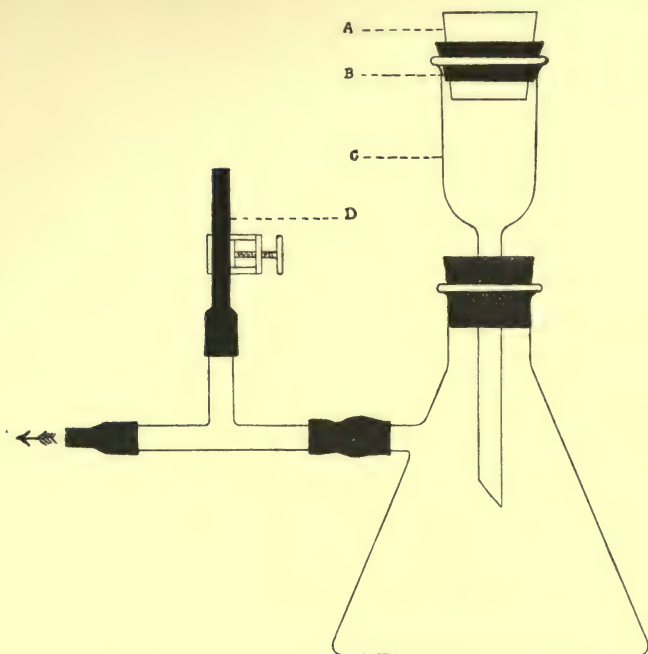


Fig. 8.

hydrochloric acid previously placed in a large gas wash bottle, and draw with the water pump a strong air current through the suspension for several minutes so as to separate the fibres. Keep the asbestos ready for use in dilute hydrochloric acid.

Fit a small Gooch crucible (A) (diameter of narrow end 14 to 18 mm.) into a glass funnel or filtering tube by means of a rubber cone or short piece of wide rubber tubing (B) (fig. 8). Pass the stem of the filtering tube through the opening of a rubber stopper inserted into a filtering flask.

Pour a little of the asbestos suspension into the porcelain Gooch

crucible, allow it to settle, apply gentle suction with the water pump, detach from the water pump, add more of the suspension, allow to settle, and again apply gentle suction. Obtain in this way a thin uniform firm layer or mat of asbestos about 2 mm. thick, covering the perforated bottom of the crucible. Place on the asbestos mat a perforated porcelain plate in order to keep it in position. Finally cover the perforated plate with a thin layer of finer asbestos, and again apply suction. The asbestos mat is then repeatedly washed with distilled water, using at first the full available pressure of the water pump, until the filtrate is quite free from particles of asbestos. Wash finally with a stream of water until the filtrate is neutral to litmus, using gentle suction so that only a slow stream of water filters through. The degree of evacuation is controlled by connecting the filtering flask with the water pump by means of pressure tubing attached to the horizontal limb of a T-tube. Fit the vertical limb of the T-tube with a piece of pressure tubing which can be compressed to a varying degree by means of an adjustable screw clip (D). The vacuum can thus be rendered more or less complete by tightening or loosening the screw clip.

When the washing has been completed, remove the Gooch crucible, place it in the concavity of the lid of a platinum crucible, cover with a porcelain or platinum lid, and dry at 100°. Then place the platinum lid on a platinum or quartz triangle and heat over the open flame for a few minutes to a red heat. Allow the crucible and platinum lid to cool in the exsiccator, and then weigh. The asbestos mat should be again washed, and the weight of the dried crucible and lids again obtained. If the weight be constant, the crucible may be used for the following estimation.

1. Determination of inorganic sulphates.

Measure 25 c.c. urine (or 50 c.c. if the urine be dilute) into an Erlenmeyer flask, add about 100 c.c. of water (or a corresponding smaller quantity if the urine be dilute), and 10 c.c. dilute hydrochloric acid. Add drop by drop 10 c.c. of 5 per cent. barium chloride solution without shaking or stirring the urine. After half an hour, shake up the solution thoroughly, allow to stand for 1 to 24 hours, according to convenience, and filter through a Gooch crucible (previously weighed together with platinum lid). Wash the precipitate with 250 c.c. of cold water. Place the crucible in the platinum lid, dry at 100°, then place the platinum lid on a triangle, upon it place the Gooch crucible covered with its own lid, and ignite, applying the flame of the bunsen, or better, spirit burner, to the under surface of the platinum. As a rule, about 10 minutes' ignition is sufficient, unless organic matter is present. Allow to cool in an exsiccator and weigh. The difference between this weight and that of the empty crucible with asbestos mat gives the weight of barium sulphate derived from inorganic sulphates.

2. Determination of total sulphates (inorganic and ethereal).

Measure 25 c.c. of urine into an Erlenmeyer flask, and add 20 c.c. of dilute hydrochloric acid (or 50 c.c. of urine and 4 c.c. of concentrated

hydrochloric acid), and boil the solution gently for 20 to 30 minutes, covering the mouth of the flask with a small watch-glass. Cool the flask under the water tap, and dilute the contents with water to about 150 c.c. Then add drop by drop 10 c.c. of 5 per cent. barium chloride without any shaking. Later, follow the procedure already described, filtering off the barium sulphate precipitate through the same Gooch crucible. Deduct the original weight of the Gooch crucible (i.e. weight of crucible plus BaSO_4 derived from inorganic sulphates) from the final weight, the result is the weight of BaSO_4 derived from the total sulphates in 25 or 50 c.c. of urine.

Deduct the result of 1 from that of 2, the value obtained is the weight of BaSO_4 derived from ethereal sulphates.

Calculation.—All results may be stated in terms of sulphur (or of SO_3 or of H_2SO_4). 1 mol. BaSO_4 (m.w. 233) corresponds to 1 mol. sulphur (m.w. 32). Let x = unknown weight of sulphur (derived from inorganic, total, or ethereal sulphates), and k be the found weight of barium sulphate. Then $x = \frac{32k}{233} = 0.1374k$.

x (stated in terms of SO_3 or H_2SO_4) = $0.343k$ or $0.4206k$.

Note.—If a Gooch crucible is not available, ash free "barium sulphate" filter paper may be used.

3. Estimation of the total sulphur of the urine (inorganic and ethereal sulphate, and neutral or unoxidised sulphur)— Denis' modification of Benedict's method.

Measure 25 c.c. of urine into a porcelain basin (4.5 inches diameter), and add by means of a pipette 5 c.c. of a solution containing 25 grms. copper nitrate, 25 grms. sodium chloride, and 10 grms. ammonium nitrate in 100 c.c. water. Evaporate to dryness on the water bath or with a very small flame, then heat gently with a small flame, gradually increasing the flow of gas until the dish is heated to redness, and continue to heat at the latter temperature for 10 to 15 minutes. Allow to cool, and add 10 to 20 c.c. of 10 per cent. hydrochloric acid. On gently warming for a few minutes a clear solution is obtained.

Transfer quantitatively to a 200 c.c. Erlenmeyer flask, make up to 100 or 150 c.c. with water, heat to boiling, and add drop by drop 25 c.c. of 10 per cent. barium chloride. Let stand one hour or more, and filter on a tared Gooch crucible. Since copper nitrate frequently contains traces of sulphate, a blank estimation should be made with the reagents, and the amount of sulphate thus found (if any) deducted in the final calculation.

Note.—Since gas contains sulphur compounds, a spirit flame is preferable to a bunsen for ignitions.

B. VOLUMETRIC METHOD.

The benzidine method¹ has been applied to the estimation of the sulphates of the urine by Rosenheim and Drummond.

¹ Müller, Raschig, and others.

"Principle of the Method.—The inorganic sulphates are precipitated from the faintly acidified urine as benzidine sulphate by means of a solution of benzidine ($\text{NH}_2\cdot\text{C}_6\text{H}_4\cdot\text{C}_6\text{H}_4\cdot\text{NH}_2$) in hydrochloric acid. As benzidine is a weak base, its salts with acids are readily dissociated, and the sulphuric acid contained in benzidine sulphate may be quantitatively titrated with standard alkali solutions, using phenolphthalein as an indicator."

"The Preparation of the Benzidine Solution.—4 grms. of benzidine are rubbed into a paste with about 10 c.c. of water, and transferred with about 500 c.c. of water into a 2 litre flask. 5 c.c. of concentrated hydrochloric acid (sp. gr. 1.19) are added, and the solution made up to 2 litres with distilled water. 150 c.c. of this solution, which keeps indefinitely, are sufficient to precipitate 0.1 gm. H_2SO_4 ."

METHOD—(1) Estimation of Inorganic Sulphates.—Measure 25 c.c. of urine into a 250 c.c. Erlenmeyer flask, and acidify with dilute hydrochloric acid (1:4) (about 1 to 2 c.c.) until the reaction is distinctly acid to Congo red. Run in 100 c.c. of the benzidine solution, allow the precipitate to settle for ten minutes, filter under pressure through a well-fitted filter paper placed on a small perforated porcelain plate fitted into a funnel of about 6 cm. diameter (taking care that the precipitate is never sucked dry), and wash out original precipitation flask with about 20 c.c. of water, previously saturated with benzidine sulphate, filtering the washings through the paper. Then transfer precipitate and filter paper with about 50 c.c. water to the original precipitation flask, shake vigorously so as to disperse the precipitate completely in the fluid, heat to the boiling point, and titrate while hot, with N/10 KOH, after the addition of a few drops of a saturated alcoholic solution of phenolphthalein. 1 c.c. of N/10 KOH = .0049 gm. H_2SO_4 .

(2) Estimation of Total Sulphates (inorganic and ethereal).—Measure 25 c.c. of urine, about 20 c.c. of water, and 2 to 2.5 c.c. of dilute HCl (1:4) into a flask, and boil gently for 15 to 20 minutes. Cool the solution, and follow the procedure already described in (1).

(3) The ethereal sulphates are represented by the difference between the total and inorganic.

More recently, Drummond has applied the method to the determination of sulphates in small quantities (e.g. 2 c.c.) of urine, and has also proved that the total sulphur of the urine (inorganic, ethereal, and neutral) may be determined by this method, if the urine be first oxidised by Benedict's method ("Biochem. J.," vol. viii. p. 143; vol. ix. p. 492).

8. Estimation of calcium and magnesium in urine (M'Crudden's method).

SOLUTIONS REQUIRED.

(a) 2.5 per cent. oxalic acid solution; (b) 20 per cent. sodium acetate solution; (c) 0.5 per cent. ammonium oxalate; (d) N/10 potassium permanganate solution.

(1) If the urine be alkaline or neutral, acidify it with a few drops

of concentrated hydrochloric acid, mix thoroughly, and filter. Measure with a pipette 200 c.c. of the filtrate into an Erlenmeyer flask. Render the urine just alkaline with ammonia, then just acid with hydrochloric acid, and add a further 10 drops of concentrated hydrochloric acid. Then add 10 c.c. of 2.5 per cent. oxalic acid, mix thoroughly, and run in slowly with stirring 8 c.c. of 20 per cent. sodium acetate solution. Allow to stand at room temperature overnight, or shake vigorously for 10 minutes; the former procedure is preferable. Filter off the precipitate of calcium oxalate on a small ash-free paper, and wash flask and precipitate free from chlorides with 0.5 per cent. ammonium oxalate solution. The precipitate may then be dried in a tared crucible, ignited and weighed as calcium oxide, or the quantity of calcium may be determined volumetrically.

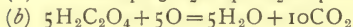
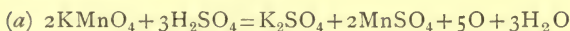
(2) Volumetric Method.—Wash out flask and precipitate thrice with water, and if the precipitate be not pigmented proceed in the way described below. If the precipitate be pigmented, it contains uric acid, and the gravimetric method should be employed (see note 2).

Perforate the bottom of the cone of filter paper with a glass rod, and wash the precipitate of calcium oxalate into the flask. Add about 50 c.c. of water and 10 c.c. of concentrated sulphuric acid, and titrate the fluid while hot with N/10 potassium permanganate to a faint red colour persisting for at least a minute.

Calculation.—One c.c. of N/10 permanganate solution is equivalent to 0.0028 grm. of calcium oxide or .002 grm. of calcium.

Notes.—(1) Preparation of N/10 potassium permanganate solution. Place 3.16 grms. of pure potassium permanganate in a litre flask, dissolve in water, and make up to a litre. The accuracy of this N/10 solution should be controlled by titrating N/10 solution of oxalic acid containing 6.3 grms. of recrystallised oxalic acid ($\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) in a litre.

PRINCIPLE OF TITRATION.



2 mols KMnO_4 (2×158 grms.) yield 5O. Therefore 2×15.8 grms. of potassium permanganate yield one equivalent of oxygen (8 grms.), which in its turn is equivalent to 45 grms. of anhydrous, or 63 grms. of crystalline oxalic acid, or to 20 grms. of Ca, or to 28 grms. of calcium oxide. A normal solution of potassium permanganate therefore contains 31.6 grms. of the salt in a litre, and N/10 solution 3.16 grms. of the salt in a litre.

(2) The following alternative method may be employed instead of perforating the filter paper and washing through the calcium oxalate. Heat a little dilute sulphuric acid (1 part by volume of concentrated sulphuric acid mixed with 19 parts water), and wash the precipitate on the filter paper 5 times. Dilute the filtrate up to about 50 c.c., cool, add 10 c.c. concentrated sulphuric acid, and titrate

in the way already described. The dilute sulphuric acid decomposes the calcium oxalate into calcium sulphate and oxalic acid which pass into the filtrate. Any uric acid which may be present is largely retained by the filter paper. Some uric acid, however, is dissolved by the dilute sulphuric acid, and therefore passes into the filtrate. Consequently the volumetric method is less accurate, although more convenient, than the gravimetric one.

Estimation of magnesium in urine (M'Crudden).—Transfer the filtrate and wash water obtained in the first stage of the determination of calcium to a porcelain basin, add 20 c.c. concentrated nitric acid, and slowly evaporate to dryness in the draught chamber. Cautiously heat the residue over the open flame until it fuses, allow to cool, add a little water, and sufficient hydrochloric acid to form a clear solution on heating. Cool and add ammonia drop by drop with constant stirring until the mixture is alkaline. Then add gradually 25 c.c. dilute ammonia (sp. gr. 0.96), stirring constantly, and allow to stand overnight. Filter and wash free from chlorides with alcoholic ammonia solution (1 pt. alcohol, 1 pt. dilute ammonia, and 3 pts. water). Dry the filter paper containing the precipitate of ammonium magnesium phosphate in the drying oven at 100° to 105°, and ignite in a weighed platinum crucible. The ammonium magnesium phosphate is converted into magnesium pyrophosphate ($Mg_2P_2O_7$).

Calculation.—Multiply the weight of magnesium pyrophosphate by 0.3624, the result gives the quantity of magnesium as magnesium oxide present in 200 c.c. of the urine analysed.

The reader is referred to a paper by Cahen and Hurtley (1916), ("Biochem. J.," vol. x. p. 308), for another method of estimating calcium in the urine and tissues.

9. Ammonia.

(1) The method of Krüger-Reich-Schittenhelm.

REAGENTS REQUIRED.

(a) Sodium chloride (solid); (b) anhydrous sodium carbonate; (c) ethyl alcohol; (d) N/10 sulphuric acid; (e) N/10 caustic soda; (f) methyl red solution.

Measure 10 to 30 c.c. of N/10 H_2SO_4 into the Peligot tube (B) (vertical limbs about 10 inches long, $1\frac{1}{4}$ to 2 inches wide, total capacity about 300 c.c.). Arrange the apparatus as shown in figure. The Peligot tube may be immersed in a vessel containing ice and water, and, under these conditions, the condenser (C) may be omitted. When a condenser is introduced, a small one (e.g. "King's College pattern") may be used.

Measure 25 to 50 c.c. urine with a pipette into flask (A), which is placed in an empty water bath, add 10 grms. sodium chloride, and 1 to 2 grms. sodium carbonate (sufficient to render urine distinctly alkaline to litmus), and close the flask with stopper. Close the stop-

cock of the separating funnel, connect E with the water pump, placing an open screw clip on the rubber tube connection, and evacuate. Fill the water bath, in which A is placed, with water at 40° to 45° , and maintain at this temperature with a small flame. The urine in A commences to boil as soon as the pressure has been reduced to 20 to 30 mm. Hg.

Add gradually from the separating funnel 10 c.c. of ethyl alcohol, stopping the addition whenever frothing becomes excessive. The boiling of the solution may also be controlled by means of the screw clip on the rubber pressure tubing attached to E. The bulb on the delivery tube from A should have a trap to prevent any of the solution being carried over. Add gradually 30 to 40 c.c. of alcohol in all, and about 10 c.c. of water to compensate for evaporation. All the ammonia passes over, and is absorbed by the acid in B within 20

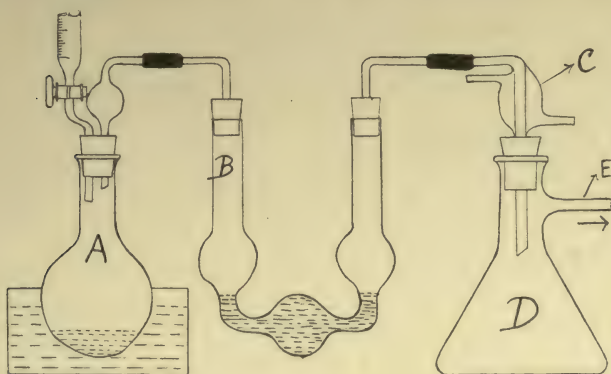


Fig. 9.

minutes from the commencement of boiling. It is advisable to allow the solution to boil for 30 minutes to render complete absorption of the ammonia certain.

Close the screw clip on the rubber tubing attached to E, and slowly admit air by gradually opening the stopcock of the separating funnel, detach B, and quantitatively transfer its contents to an Erlenmeyer flask, washing out the tube at least three times with water. If any of the N/10 acid have passed into D, wash out D, and the connecting tubes in a similar way and transfer the washings to the Erlenmeyer flask. If necessary, add a few more drops of methyl-red solution, and titrate with N/10 NaOH until the red colour of the indicator is just discharged or becomes light yellow.

Calculation.—20 c.c. of N/10 H_2SO_4 already partially neutralised with absorbed ammonia required x c.c. of N/10 NaOH for complete neutralisation. Hence the quantity of ammonia in 25 c.c. of urine is $(20 - x) \times 1.7$ mgrms., or the quantity of ammonia—N is $(20 - x) \times 1.4$ mgrms.

PRINCIPLE OF THE METHOD.

If urine be heated with caustic alkali at atmospheric pressure, both the preformed ammonia and ammonia derived from the partial hydrolysis of urea are set free. If the distillation be carried out *in vacuo* at a lower temperature, *e.g.* 40° to 45° , and a weaker alkali (*e.g.* sodium carbonate) be used, no hydrolysis of urea occurs and only the preformed ammonia is set free. The alcohol vapour carries over the ammonia with it, and ensures the complete transference of the ammonia from the distillation flask and connecting tubes to the N/10 acid.

This method gives accurate results, and, if .02N solutions be used, it can be applied to the estimation of ammonia in smaller quantities of urine (*e.g.* 5 c.c.). The standard alkali should be free from carbonate.

Folin's "aeration" method will be described later under "Urea."

Note.—The urine should be fresh, and a little toluene should be placed in the vessel in which it is collected.

ORGANIC CONSTITUENTS.

1. Total nitrogen.

This method may be taken along with those concerned with the organic constituents, as much the larger proportion of the nitrogen is organically bound. The method that is practically always employed for the estimation of nitrogen in such fluids as urine, milk, etc., is that of Kjeldahl, and although it is far from being free from fallacies, it is the only practicable method at the present day in the case of such mixed fluids as the above-mentioned ones. It cannot be employed if nitro- or pyridin-derivatives are present. If nitrates are present, they require to be reduced to ammonia. The principle of the method is by no means clear. The urine is boiled with concentrated H_2SO_4 , and certain substances are added which either aid the incineration by raising the boiling point of the acid, or by acting as oxidising agents.

The duration of the incineration is much lessened by the addition of such oxidising agents as anhydrous cupric sulphate, mercuric oxide, or metallic mercury. The last-mentioned one is, in many respects, the best, as it shortens the process very much. It forms, however, mercuri-ammonium compounds, which require to be broken up before distillation with NaOH can set the ammonia free. This can be satisfactorily done by adding zinc dust to the acid fluid

after the greater part of the acid has been neutralised. This results in the evolution of hydrogen, and this *in statu nascendi* effects the decomposition, and afterwards prevents bumping in the alkaline fluid. This decomposition is most usually brought about by adding alkaline sulphide, which, however, is a more troublesome method than the one just described.

In the case of urine, the addition of K_2SO_4 and $CuSO_4$ to the sulphuric acid accomplishes the oxidation sufficiently rapidly, and thus renders either of the above additions unnecessary. This addition of K_2SO_4 (Gunning) is very useful, as it forms acid salts with the sulphuric acid, which, unlike the acid itself, give up their water more readily than they do the acid, and so overcome the tendency to gradual weakening of the acid fluid from loss of H_2SO_4 .

If there be much tendency to frothing during the incineration, it is advisable to add a small piece of paraffin to the liquid. After complete oxidation of the carbon, etc., the nitrogen is present in the form of $(NH_4)_2SO_4$, and from this the ammonia is set free by making the solution strongly alkaline with KOH , and distilling the ammonia over into a measured quantity of $\frac{N}{5} H_2SO_4$.

The amount of ammonia, and from it the nitrogen, is calculated by determining the number of c.c. of the acid which have been neutralised.

Process.

From the twenty-four hours' urine take up in a pipette 5 c.c., run into round-bottomed Kjeldahl flask of about 500 c.c. capacity, add 10 c.c. conc. H_2SO_4 and three or four crystals of $CuSO_4$. Place over a small gas flame in draught chamber, supporting the flask in inclined position, and boil until all the water has evaporated, then add about 5 grms. of pure K_2SO_4 (which subsequently acts as the pyrosulphate). Place small funnel or tube with bulb upon it in neck of flask, and heat the mixture at first very carefully and then strongly. Boil for some time after the

mixture has become quite clear (at least half an hour). Now remove the flame and allow to cool slightly, then pour

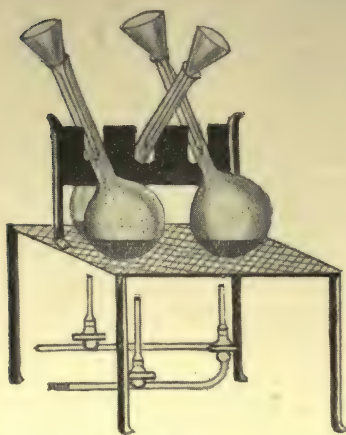


Fig. 10.

Incineration Apparatus (Kjeldahl).

in about 50 c.c. of distilled water, and shake thoroughly. Add about half a teaspoonful of talc to prevent bumping; pour about 20 c.c. 33 per cent. KOH or NaOH into separating funnel, which is inserted in one of the openings of the bored rubber stopper in the distillation flask,¹ and gradually run in the alkali until the fluid is almost neutralised, although still of slight acid reaction. Before rendering the solution strongly alkaline, see that all

necessary connections are made for the distillation. Through the other opening in the stopper, insert a suitable bent glass tube which has upon it a safety bulb to prevent fixed alkali sparking over into the condensing tube. Connect this with a Liebig's condenser (a water-jacket, although not absolutely necessary, is of advantage). This is connected at the other end with an adapter which passes down into the receiving flask containing 50 c.c. $\frac{N}{5}$ H_2SO_4 and a few drops of methyl red solution (.02 per cent. alcoholic solution). Before commencing to distil, see that the end of the adapter dips into the acid. It is very advisable to connect with the receiver a bent glass tube with bulb, containing glass beads moistened with a measured quantity of the $\frac{N}{5}$ H_2SO_4 . Now, when all connections are made, run in from the

¹ The same flask that was used for the incineration is employed for the distillation.

separating funnel sufficient KOH^1 or NaOH (about 50 c.c. in all) to make solution in distillation flask strongly alkaline. The mixture has now a deep blue tint, due to the formation of ammonium cupric hydrate. Close stop-cock immediately after the addition of the necessary amount of alkali. Place the flame below the flask, and boil until the distillate is free from ammonia. Care should be taken to prevent, as far as possible, much condensation on the walls of the distillation flask, as otherwise some fixed alkali may be carried over by the condensed water vapour. Therefore surround safety bulb, etc., with cotton-wool. However much care is taken, there is a tendency, if the distillation be prolonged, to have some fixed alkali carried over. Hence it is advisable not to lay too much stress upon very slight alkaline reaction of the distillate after prolonged distillation.

When the distillate no longer gives an alkaline reaction with litmus paper, remove the flask and titrate the distillate against $\frac{N}{5}$ NaOH . Knowing the amount of acid taken at the outset, and the acidity of the distillate remaining after all the ammonia has been fixed, the amount of acid, which has been neutralised, is arrived at.

$$\text{Now, 1 c.c. of } \frac{N}{5} \text{ H}_2\text{SO}_4 = 1 \text{ c.c. } \frac{N}{5} \text{ NH}_3,$$

$$\text{and 1 c.c. } \frac{N}{5} \text{ NH}_3 = \frac{17}{5} \text{ mgrms. NH}_3, \text{ or } \frac{14}{5} \text{ mgrms. N.}$$

Example.

Originally, 50 c.c. $\frac{N}{5}$ H_2SO_4 .

After distillation, excess of acid neutralised by

$$30 \text{ c.c. } \frac{N}{5} \text{ NaOH} = 30 \text{ c.c. } \frac{N}{5} \text{ H}_2\text{SO}_4 ;$$

\therefore 20 c.c. $\frac{N}{5}$ H_2SO_4 have been neutralised by an amount

¹ The tendency to "bumping" is less with KOH than with NaOH (Bang).

of NH_3 equivalent to 20 c.c. $\frac{\text{N}}{5} \text{NH}_3 = .0028 \times 20$ grm. nitrogen. 5 c.c. of urine therefore yielded .056 grm. of nitrogen or 1.12 per cent. If 1500 c.c. of urine were passed in twenty-four hours, the total quantity of nitrogen is $1.12 \times 15 = 16.8$ grms.

Notes.—(1) A number of modifications of Kjeldahl's method have been proposed. The incineration may be hastened by the addition of more powerful oxidising agents, but this procedure involves care to avoid loss of nitrogen from decomposition of ammonia, and is therefore unsuitable for class work.

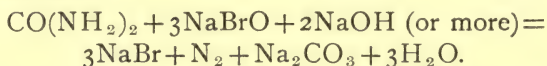
Steam and alcohol distillation methods, and aeration methods have also been employed to facilitate the second stage of the process.

(2) When a sensitive indicator, such as methyl red is used, accurate results can be obtained with smaller quantities of urine, *e.g.* 1 to 2 c.c. (accurately measured). 0.02 N to 0.1 N solutions of alkali (free from carbonate) and acid may then be used for the titration.

2. Urea.

(A) HÜFNER'S METHOD.

This is the method which is most commonly employed in clinical work. It depends upon the decomposition of urea into water, carbonic acid and nitrogen by sodium hypobromite. The strongly alkaline solution absorbs the CO_2 , and thus the only gas remaining is nitrogen, the volume of which can be estimated.



Theoretically, 1 grm. urea gives off 372 c.c. nitrogen at 0° and 760 mm. Hg pressure, but the hypobromite solution only sets free 354.33 c.c. nitrogen from each gramme. It is this latter factor which is taken for the calculation. There are other bodies in the urine which give off their nitrogen, either in whole or in part, under the same conditions, so that this introduces another error. As a rule the volume is not corrected for temperature and pressure.

Method.

Pour 25 c.c. NaBrO solution¹ into a wide-necked

¹ *NaBrO solution.*—Dissolve 100 grms. NaOH in 250 c.c. water, and add 25 c.c. bromine after the solution has been completely cooled. This must be done in well-ventilated draught chamber.

Erlenmeyer flask, or into a suitable bottle. Take up 5 c.c. protein-free urine in pipette, run into small tube, and carefully introduce the latter into the vessel containing the hypobromite, placing it against side of flask. Insert rubber stopper in flask and connect by suitable tubing with one limb of a T-piece, which has on the upper limb a piece of tubing with a clip, while the lower limb is connected with an inverted burette in a tall jar of water.¹ Open the clip and raise or lower the tube until the fluid stands at about the level of the uppermost graduation. Now clamp tube, and test for possible leakage by raising and lowering the burette, thus varying the pressure. If any leakage does occur, localise its position by closing different parts of tubing, testing stopper, etc. Now, having seen that no air can escape from the system, especially seeing that stopper is securely in, take the reading of the burette when the water is at the same level in the burette and in the

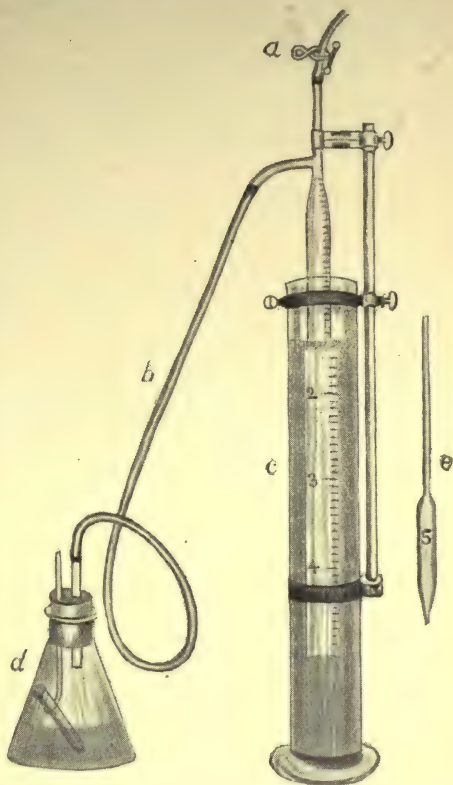


Fig. 11.—Dupré's Ureometer.

¹ An apparatus, such as the one shown in the figure (Dupré's), may be employed.

outer vessel. Now tilt the flask so as to allow the urine to mix with the alkaline hypobromite solution, seeing that the small tube is thoroughly washed out with the latter. The nitrogen comes off rapidly. Allow the gas to return to the original temperature, hastening this by placing the flask in a dish of cold water.¹ Now take the reading again with the water in and outside at the same level. The volume of the gas being thus obtained, the amount of urea in the urine is easily calculated.

Example.

40 c.c. nitrogen were obtained from 5 c.c. urine, and
35.433 c.c. nitrogen are obtained from .11 grm. urea.
∴ 40 c.c. nitrogen = *circa* .11 grm. urea, *i.e.* in 5 c.c. urine.

If 1500 c.c. urine were passed in twenty-four hours, then $.11 \times 300 = 33$ grms. urea secreted in twenty-four hours.

Or, if a correction for temperature and pressure be made, then the calculation is made as follows :—

$$W = \frac{1500V(b-b')}{760 \times 354.33 \times 5(1+.003665t^{\circ})}$$

when—

W = grms. urea in 1500 c.c. urine (twenty-four hours' quantity).

V = volume of nitrogen in c.c.

b = observed barometric pressure in mm. Hg.

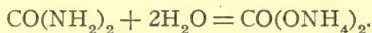
b' = tension of aqueous vapour at t° .

t° = temperature at time of estimation.

5 = c.c. of urine employed.

(B) The "Urease" Methods of Estimating Urea.

These methods are based on the fact that an enzyme urease, present in soya bean, rapidly converts urea into ammonium carbonate, thus :—



The ammonia formed may be determined by direct titration, or fixed

¹ The water should be kept in the vessel at room temperature overnight. Water taken direct from the tap should never be used for cooling the flask.

alkali (sodium carbonate) may be added, and the ammonia carried over into a known quantity of N/10 acid by a current of air. The amount of acid neutralised by ammonia can then be determined by titration with N/10 caustic soda. The ammonia may also be distilled off at 45° *in vacuo*.

0.5 to 1 grm. of finely powdered soya bean may be used for each estimation, or a purified preparation of the enzyme may be made.

NOTE.—Preparation of urease (Van Slyke and Cullen).

Extract 1 part of finely powdered soya bean with 5 parts of water at room temperature for one hour, and clear the solution by centrifugation or filtration through paper pulp.

Pour this extract slowly into 10 times its volume of acetone. Filter off the precipitate, dry *in vacuo*, and powder. The dry powder retains its activity indefinitely.

Active commercial preparations are obtainable from the Arlington Chemical Company, and from Hynson Westcott, and Dunning, Baltimore. The latter preparation (the only one which the writers have tried) is very active.

An active solution may be obtained by dissolving 2 grms. of the dry powder (prepared by Van Slyke and Cullen's method) in 10 c.c. of a solution containing 0.6 grm. of K_2HPO_4 , and 0.4 KH_2PO_4 per 100 c.c.

(1) Marshall's Method.

Measure two 5 c.c. portions of M/5 urea solution or urine into bottles (about 250 c.c. capacity) provided with glass stoppers. Grind one or two tablets (Dunning) in a mortar with a little water, and filter the extract. Wash out the mortar repeatedly with water, using about 100 c.c. in all, filter, and add the total filtrate, which is slightly opalescent, to one sample of the urea solution or urine. An alternative method is to add 1 c.c. of the 10 per cent. urease solution prepared by Van Slyke and Cullen's method. Dilute the other sample of urine with the same volume of water (about 125 c.c.) without adding urease.

Securely stopper both bottles, and place them in the incubator at 37° to 40° for $1\frac{1}{2}$ to 2 hours. (An alternative method is to add a few drops of toluene to each solution and leave at room temperature for 5 to 8 hours.) Then remove the bottles from the incubator, cool thoroughly under the tap, and add the same number of drops of methyl orange (or better, methyl red) solution to each. Then titrate each solution with N/10 or N/5 HCl to a distinct pink, matching the tints as closely as possible. Since much more acid is required for the urine or urea solution, acted on by urease, than for the control, an equivalent amount of water may be added to the control.

A few c.c. of the enzyme solution, or of an aqueous extract, of one or two tablets should also be titrated to determine the quantity of N/10 acid required to neutralise the quantity of urease solution

used for the estimation. The amount of $N/10$ acid required for neutralisation of the urease solution is usually exceedingly small.

CALCULATION.

Let x = number of c.c. of $N/10$ acid required for the titration of the urea solution or urine acted on by urease, k the number of c.c. of acid required for the control, and m the number of c.c. acid required for the enzyme solution. Then $(x - k - m) \times .003$ gram. is the quantity of urea in grammes in 5 c.c. of the urine or urea solution.

This method gives excellent results, and is probably the one best adapted for clinical work. The writers find the end point for the titration more sharply defined with methyl red as indicator instead of methyl orange. The error of observation does not appear to exceed 2 per cent.

When glucose is present, this method yields less accurate results, and the aeration method may replace it in such cases.

The activity of urease preparations, or the crude soya bean meal, should always be tested on a solution of urea of known concentration. One thus learns whether the preparation is active or not, and what time is required for complete hydrolysis of the urea.

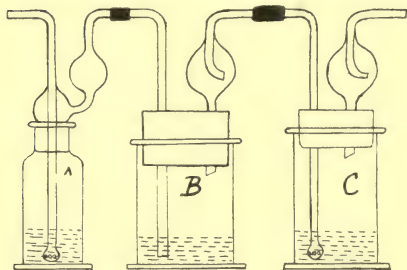


Fig. 12.

(2) **Aeration Method.**
(Plimmer and Skelton's procedure. The "Biochemical Journal," vol. viii. p. 70.)

Arrange the apparatus shown in fig. 12. Place dilute sulphuric acid in the cylinder (A), and 50 c.c. of $N/10$ sulphuric acid coloured with a few drops of methyl red (or alizarin red or methyl orange) solution in the absorption cylinder C. Measure 5 c.c. of urine or urea solution of known concentration and 50 to 60 c.c. of water into B (the cylinder should be higher than that shown in the figure, about 25 cm.). Add 0.5 to 1 gram. of powdered soya bean, and about 2 c.c. of liquid paraffin (B.P.) to prevent frothing. Connect the parts of the apparatus with rubber tubing, and the outlet tube of (C) by pressure tubing with the water pump. Place (B) in a water bath at 40° , and draw a current of air through the apparatus for one hour. Stop the suction, disjoint the apparatus, add 1 or 2 grms. of anhydrous sodium carbonate to the solution in B to set free ammonia fixed as an ammonium salt. Again make the connections and aspirate a slow current of air for a few minutes and then a strong current through the apparatus for $\frac{1}{2}$ to 1 hour. Then disjoint the apparatus and titrate the excess of acid in C with $N/10$ caustic soda. Part of the 50 c.c. of $N/10$ acid has been neutralised by the preformed ammonia of the urine, and part by ammonia derived from the hydrolysis of

urea. A separate estimation of the preformed ammonia by either the "vacuum" or "aeration" methods must be carried out before the quantity of urea can be determined.

CALCULATION.

1 mol of urea (60 grms.) yields 34 grms. (2 mols) of ammonia. Hence, .003 grm. of urea are equivalent to .0017 grm. of ammonia or 1 c.c. of $N/10 \text{ NH}_3$.

Let x = number of c.c. of $N/10$ caustic soda required to neutralise the free acid in (C), then $50 - x$ is the number of c.c. of $N/10$ acid, neutralised by ammonia. Let k = number of c.c. of $N/10$ preformed ammonia found in 5 c.c. of the same urine by a previous estimation, then the quantity of urea in grammes present in 5 c.c. of urine is $(50 - x - k) \times .003$.

Notes.—(1) When a simple gas washing bottle is used for (C), it is advisable to have a second one containing a known quantity of $N/10$ acid so as to retain any ammonia which may have escaped absorption by the $N/10$ acid in the first. Allihn's gas wash bottle or the most recent form of Folin's gas wash bottle may be used with advantage instead of C.

(2) In order to ensure complete transference of ammonia to the absorption cylinder (C), the apparatus may be partially evacuated by attaching a piece of pressure tubing to the inlet tube of (A) and partially obliterating its lumen with a screw clip, while suction is maintained. Finally, the pressure of the screw clip should be gradually released in order to prevent too rapid entrance of air.

(3) Other methods of procedure have also been devised.

Van Slyke and Cullen's method of estimating the urea and preformed ammonia in small quantities of urine.

In this method, the cylinders B and C are replaced by boiling tubes (200 mm. by 25 to 30 mm.), and the outlet tubes of (B) and (C) have small bulbs (broken 5 c.c. pipettes may be used). 25 c.c. of $N/50$ sulphuric acid is placed in C. Measure 0.5 to 1 c.c. of urine with an accurate Ostwald pipette (i.e. one which delivers the amount stated, when blown out, after draining for 15 seconds. Ostwald-Luther's "Physiko-chemische Messungen," 3rd ed., pp. 166-170) into tube (B), add 2 c.c. of soya bean extract, 3 c.c. of water, and one or two drops of caprylic alcohol to prevent frothing. Close and connect the tubes (B) and (C) with one another and with a gas wash bottle (A) containing 1 in 10 sulphuric acid. Immerse B in water at 45° , and leave about 15 minutes, maintaining a slow current of air through the apparatus with the water pump. Then aspirate a rapid current of air through the apparatus for 1 or 2 minutes. Stop the current, remove the stopper of (B), taking care that no fluid is lost, add 4 to 5 grms. of anhydrous potassium carbonate, aspirate a slow current of air through the apparatus for 2 minutes, and a rapid one for 15 minutes. Disjoint the apparatus, add a few drops of methyl

red or alizarin red to the acid in (C) and titrate the free acid with N/50 caustic soda.

CALCULATION.

Let x = number of c.c. of N/50 NaOH required for neutralisation, then $(25 - x) \times .00028$ = grammes of preformed NH_3 -nitrogen plus urea-nitrogen in 0.5 or 1 c.c. of urine.

(4) (a) **Estimation of the preformed Ammonia by Folin's "Aeration Method."**—The same apparatus (fig. 11) may be used for the estimation of the preformed ammonia of urine as that employed for the estimation of urea by Plimmer and Skelton's method.

Place 20 c.c. N/10 sulphuric acid in the cylinder (C). Measure 25 c.c. of urine into B, add 1 to 2 grms. anhydrous sodium carbonate, and a few drops of liquid paraffin or caprylic alcohol to prevent frothing. Arrange the apparatus, and draw a rapid current of air through it for $1\frac{1}{2}$ to 2 hours.

CALCULATION.

Let x be the number of c.c. of N/10 NaOH required to neutralise the free acid in (C), after the ammonia has been absorbed, then $(20 - x) \times .0017$ are the grammes of ammonia in 25 c.c. of urine, or $(20 - x) \times .0014$ are the grammes of NH_3 -nitrogen in 25 c.c. of urine.

(b) **Estimation of preformed Ammonia (Van Slyke's Method).**—The same apparatus is used as for Van Slyke and Cullen's method for determining urea.

Measure 20 c.c. of N/50 sulphuric acid into tube (C). Measure 5 c.c. of urine into B, add 2 drops of caprylic alcohol and 4 to 5 grms. of anhydrous potassium carbonate. Connect up the apparatus, aspirate a slow current of air for 2 or 3 minutes, and a rapid current of air for about 15 minutes. Disjoint C, loosen the stopper, wash down the inlet and outlet tubes, and walls of absorption tube, and titrate the free acid with N/50 caustic soda solution, using methyl red as indicator.

CALCULATION.

Let k be the number of c.c. of N/50 NaOH required to neutralise the free acid.

Hence $(20 - k) \times .00028$ gives the quantity of NH_3 -nitrogen in grammes in 5 c.c. of urine. The total preformed NH_3 -nitrogen and urea-nitrogen in 5 c.c. of urine are obtained by the urease aeration methods. Hence, if the NH_3 -nitrogen in 5 c.c. of urine be deducted from this total, the quantity in grammes of urea-nitrogen in 5 c.c. of urine is obtained.

(5) Method of estimating urea by distillation in vacuo.

The ammonia derived from the hydrolysis of urea by urease may also be determined by distillation *in vacuo* with the aid of the Krüger-

Reich-Schittenhelm apparatus. According to the writers' experience this is the most accurate and reliable method.

PROCEDURE.—*Accurately measure with an Ostwald pipette 2 c.c. of urine into the distilling flask. Grind up a urease tablet in a mortar, and wash into the flask with about 25 c.c. of water. Assemble the apparatus described on p. 310, placing 25 c.c. of N/10 sulphuric acid in the Peligot tube. Immerse the flask in a water bath at 40°, reduce the pressure of the system slightly by means of the water pump, and shut off the apparatus from the water pump by tightening a screw clip or closing a glass stopcock inserted by means of pressure tubing between the filtration flask and the water pump. Allow the urease to act for an hour. Then set the water pump in action and gradually release the clip or open the stopcock. Part of the ammonia then distils over into the acid in the Peligot tube. Dissolve 3 to 4 grms. of anhydrous sodium or potassium carbonate in water, and run the solution through the separating funnel into the distillation flask. The rest of the procedure is the same as that described on pp. 310 and 311, 15 c.c. of a saturated solution of sodium chloride being added through the separating funnel in place of the solid.*

The calculation is the same as that for the "aeration" method, the result obtained including both urea-N, and preformed ammonia-N.

3. Uric Acid.

The method most frequently used for the estimation of uric acid is based on its complete precipitation from the urine as an ammonium salt by the addition of ammonium chloride (30 grms. for each 100 c.c. of urine) and ammonia. The precipitate is then filtered off, washed free from chlorides (which reduce potassium permanganate in acid solution) with an ammonium sulphate solution, and the amount of uric acid set free by dilute sulphuric acid determined by titration with N/20 potassium permanganate solution. This procedure, which was devised by Hopkins, is the standard method, but requires a considerable expenditure of time. A simplification of the method due to Folin and Shaffer has largely replaced the original procedure. Cole has recently proposed a modification of Hopkins' method, which can be rapidly carried out, and may, in its turn, replace that of Folin and Shaffer ("Practical Physiological Chemistry," pp. 341 to 344). Folin and Shaffer's method is described here, since the writers have not yet acquired an adequate knowledge of the technique of Cole's method, and the Folin-Shaffer method also gives good results.

REAGENTS REQUIRED.

(a) 500 grms. ammonium sulphate, 5 grms. uranium acetate, 60 c.c. 10 per cent. acetic acid, and 650 c.c. water. The volume of the solution is about 1 litre.

(b) 10 per cent. ammonium sulphate solution conveniently placed in a wash bottle.

(c) N/20 potassium permanganate. Place 1.581 grm. of chemically pure potassium permanganate in a litre flask, dissolve in water and make up to a litre. This solution should be standardised by titration against N/20 oxalic acid. 1 c.c. of N/20 potassium permanganate is equivalent to 0.00375 grm. of uric acid.

PROCEDURE.

Measure 200 c.c. of urine with a pipette into a flask, add 50 c.c. of (a), shake thoroughly, and allow to stand for about 10 minutes until the precipitate has settled. Filter through a dry filter paper into a dry flask. This treatment removes mucoid substances.

Transfer 100 c.c. of the filtrate with a pipette to a beaker, add 5 c.c. of concentrated ammonia, and allow the solution to stand for 24 hours. Decant the fluid through a small filter paper, quantitatively transfer the precipitate of ammonium urate to the filter paper by washing with solution (b), and wash the precipitate free from chlorides with solution (b). Perforate the bottom of the filter paper with a glass rod, and wash the precipitate through the funnel into the beaker by means of a jet of hot water delivered from a wash bottle, using about 100 c.c. in all. Cool, add 15 c.c. of concentrated sulphuric acid, and titrate at once (while the solution is hot, about 65°) with N/20 potassium permanganate. Gradually add the potassium permanganate solution from a burette. The red colour is at first discharged instantaneously. When a faint red colour diffuses through the body of the fluid, and remains for a second or two, the end point is reached.

CALCULATION.

If x be the number of c.c. of potassium permanganate used, then $\frac{5x \times .00375 \text{ grm.}}{4} + .003 \text{ grm.}$ is the weight of uric acid present in 100 c.c. of urine. x is multiplied by 5/4, because the quantity of filtrate taken corresponds to 80 c.c. of urine. .003 grm. is added to the result as a correction for loss of ammonium urate owing to its slight solubility in the reagents used.

Folin and Macallum have recently introduced a colorimetric method based on the colour reaction given on p. 211. The colour reaction is so delicate that only a small quantity (1-3 c.c.) of urine is required for each analysis. With certain modifications, the method can also be utilised for the estimation of uric acid in blood.

4. Creatinine.

The method of estimation will be described in a subsequent chapter.

5. Glucose.

Different methods are employed, those in most common use depending upon the reduction of cupric oxide by glucose in alkaline solution. When certain conditions are fulfilled, a fixed quantity of Fehling's solution (*vide* p. 14) is reduced by a given weight of glucose. Among these conditions the most important are the following:—The sugar solution must be between .5 and 1 per cent., must be added to the Fehling solution practically at one time, and not in small portions, and the reduction must take place at boiling point.

If one run a 1 per cent. solution of glucose into boiling Fehling's solution (diluted 1 in 5), 5.055 molecules CuO are reduced by one molecule glucose.

The urine must be free from albumin. If not, a fixed volume is brought to boiling point, acidified with acetic acid, and boiled for a very short time, until the coagulum has balled together. The urine is then made up again to its original volume by addition of distilled water and filtered through a dry filter paper.

1. Fehling's Method.

Run 10 c.c. freshly prepared Fehling's solution from a burette into a porcelain basin, dilute with 40 c.c. of water, and bring to boiling point. Now, from a burette containing glucose solution (diabetic urine) run in 1 c.c. at a time, keeping the solution at boiling point. Note when the blue colour is undoubtedly discharged and there is probably a slight yellow colour in the supernatant liquid, due to the action of the strong alkali upon the excess of glucose. This first preliminary titration gives a rough idea of how much sugar is present. If less than 5 c.c. of the glucose solution reduce the 10 c.c. Fehling completely, then it requires to be diluted until between 5 and 10 c.c. are required to arrive at the end point.

After diluting to this extent (i.e. a .5 to 1 per cent. glucose solution), repeat the titration, but in this case instead of running in the solution in small portions, run

in at once slightly less than the amount you think will produce complete reduction, and then find the end point, namely, the discharge of the blue tint in the supernatant fluid, by adding cautiously a few drops at a time. Repeat this, trying as far as possible to add at one time almost all the glucose solution that will effect the reduction.

It is very advisable, if the solution has been boiling for some time, to add a small quantity of strong alkali, which allows the deposit to settle down more rapidly.¹ The end point is best seen by tilting the basin slightly and noting the colour of the supernatant liquid when the red Cu_2O has begun to settle. After a little practice this end point can be easily and accurately determined, care being taken however to take it before the whole of the deposit has settled down, as the subsequent oxidation by the atmospheric oxygen causes the blue colour to reappear. The calculation is easily carried out. Each c.c. of Fehling equals 5 mgrms. glucose, so that when 10 are taken, the volume of urine (or diluted urine) added contains 50 mgrms. glucose. If the urine has been diluted, the correction for this dilution is made. Knowing the twenty-four hours' quantity, the daily excretion is at once arrived at.

2. Ling and Rendle's modification of Fehling's method.

The chief difficulty of the foregoing estimation is the recognition of the end point or stage at which reduction is complete, and the blue colour therefore discharged. A number of indicators have been employed to facilitate the determination of the end point. One of the best of these is Ling and Rendle's ferrous thiocyanate indicator. The application of this solution as an indicator for cupric hydrate is based on the fact that colourless ferrous thiocyanate is oxidised by cupric salts to the red coloured ferric thiocyanate.

PREPARATION OF THE INDICATOR.

Dissolve 1 grm. of ferrous ammonium sulphate and 1.5 grm. of ammonium thiocyanate in 10 c.c. of water at about 45° . Cool, add 2.5 c.c.

¹ The addition of various calcium salts has been suggested as an aid to the precipitation of the cuprous oxide. There are many disadvantages, however, associated with such a procedure which it is advisable to avoid.

of concentrated hydrochloric acid, and shake with a little zinc dust until the brownish-red solution is completely decolorised.

PROCEDURE.

Measure 10 c.c. of Fehling's solution with a pipette into a 150 c.c. flask, and dilute with about 30 c.c. of water. Dilute the urine or glucose solution until about 10 c.c., reduce 10 c.c. of Fehling's solution. (A preliminary approximate estimation may be made with urine diluted 1 in 10 with water.) Raise the diluted Fehling's solution to the boiling point and run in the diluted urine, boiling and shaking after each addition, until the blue colour is almost discharged. Place a number of drops of the indicator on a porcelain tile. Withdraw a drop of fluid from the flask with a glass rod or capillary tube, and bring it into contact with the centre of one of the drops of indicator. The appearance of a red colour indicates that reduction is incomplete. The glucose solution is again added gradually to the boiling dilute Fehling, and a drop of the solution tested against the indicator after each addition. When a red colour is no longer obtained, the end point is reached. At the final stage the solution in the flask should be boiled for 10 seconds before a drop of it is tested against the indicator. A second more rapid titration should be carried out, using the result obtained by the first as a guide. The calculation is made in the way already described.

3. Benedict's Method.

REAGENTS REQUIRED.

(1) (a) Dissolve 200 grms. sodium citrate, 200 grms. crystalline sodium carbonate (or 75 grms. of the anhydrous salt), and 125 grms. of potassium thiocyanate in hot water. Cool, transfer to a litre flask, and make up to about 800 c.c. with water.

(b) Dissolve 18 grms. of chemically pure copper sulphate (weighed to 0.1 mgrm.) in about 100 c.c. of water, and pour this solution with constant shaking into solution (1). Add 5 c.c. of 5 per cent. potassium ferrocyanide to the solution in the litre flask and mix thoroughly. Wash out the flask which contained the copper sulphate repeatedly with small quantities of water, using the wash water to make up the solution in the litre flask to one litre.

25 c.c. of this solution are reduced by .05 gm. glucose, .052 gm. fructose, .067 gm. lactose, or .074 gm. maltose.

(2) Anhydrous sodium carbonate.

PRINCIPLE OF THE METHOD.

The cuprous hydrate or oxide formed on reduction reacts with the thiocyanate to form a white precipitate of cuprous thiocyanate. The potassium ferrocyanide also helps to prevent any separation of cuprous oxide.

PROCEDURE.

Place the sugar solution (diluted if necessary so that about 10 c.c. reduce 25 c.c. of Benedict's reagent) in a burette. Measure

25 c.c. of Benedict's reagent with a pipette into a flask (about 150 c.c. capacity) or porcelain basin (25 to 30 cm. diameter), add about 5 grms. of anhydrous sodium carbonate (or 10 to 15 grms. of the crystalline salt), and heat to boiling. Then add the sugar solution somewhat slowly to the reagent (which must be kept boiling) until the blue colour is greatly lessened. Finally, add the sugar solution drop by drop until the last trace of blue colour has just been discharged. The solution in the flask must be kept boiling throughout the titration. Let k = number of c.c. of sugar solution added, and x the unknown percentage of glucose, then $x = 5/k$.

If less than 10 c.c. of the glucose solution or diabetic urine were required the original solution must be diluted, and a second estimation carried out, allowance for the extent of dilution being made in the final calculation.

4. Pavy-Fehling Method.

The difficulty in noticing the end reaction in Fehling's method is due to the red cuprous oxide not separating out sufficiently rapidly, and in this method this difficulty is overcome by adding ammonia to the solution, which keeps the Cu_2O in solution, so that the colourless solution obtained, when all the cupric salt has been reduced, is free from precipitate. As, however, Pavy's solution, on boiling, gives off so much ammonia as to render the titration, not only more or less disagreeable, but also fallacious, as the titre then alters, and as the hot colourless solution (at the end point) rapidly takes up oxygen from the air and becomes blue again, the process must be carried out in such a way as to prevent, as far as possible, ammonia coming freely off, and also to prevent the entrance of oxygen. The method is now rarely used.

The urine should be diluted until about 10 c.c. will reduce 10 c.c. of Pavy-Fehling solution, diluted with an equal volume of water.

Process.

Run into a flask of about 150 c.c. capacity 10 c.c. of Pavy-Fehling solution,¹ add an equal volume of water, and insert a double-bored rubber stopper. Fill a burette with diluted glucose solution (the suitable dilution may be arrived at by some preliminary titrations). Connect the burette by means of a short piece of rubber tubing with a pointed glass tube. Place a clip on the rubber tubing and run some of the glucose solution through the glass jet so as to drive through any air bubbles. Now pass the glass tube connected with the burette through one of the openings in the rubber stopper, so that it projects freely into the flask. Pass first through the other opening in the stopper a glass tube with a U-shaped bend upon it. It is advisable to have one or two bulbs on this tube filled with pumice stone moistened with H_2SO_4 in order to absorb the ammonia which comes off so freely.

In the first place, boil the fluid for a short time to drive the air

¹ See Appendix.

out of the flask, then take burette reading, and run into the boiling fluid the diluted urine until the blue colour is just discharged. This should be done gradually on the first occasion, a longer interval being given after each addition than with ordinary Fehling, as the reduction takes place more slowly. The subsequent control titrations are carried out in the same way as described under Fehling's method.

The calculation is easily made—

10 c.c. Pavy-Fehling = 5 mgrms. glucose.

Reduction of this quantity is effected by y c.c. of urine, diluted say 1 in 20.

Then $\frac{y}{20}$ c.c. urine = 5 mgrms. glucose.

If 5000 c.c. diabetic urine passed in twenty-four hours, then

$$\frac{5000 \times 5 \times 20}{y \times 1000} = \text{grms. glucose in twenty-four hours.}$$

Note.—A large number of other titration methods have been proposed. The following list contains references to some of the most valuable methods :—

Bertrand's method ("Practical Biological Chemistry," by Bertrand and Thomas, translated by H. A. Colwell, Bell & Son, pp. 61 to 72). Bang, "Bioch. Zeitschr.," vol. xlix. p. 1. Wood and Berry, "Cambridge Phil. Journal," xlv. p. 103. Folin and M'Ellroy, "J. of Biol. Chem.," xxxiii. p. 513; and xxxviii. p. 287. Peters, "J. Am. Chem. Soc.," xxxiv. p. 422 and p. 928.

5. By Polarimeter or Saccharimeter.

This is an exceedingly useful method. It is of special value, as furnishing confirmatory evidence as to the amount and nature of reducing substances, which, by the ordinary quantitative methods alone, could not be determined. There may be two substances present which rotate the plane of polarised light to a different extent, or in a different direction, and yet both may reduce Fehling in the same way, and so the estimation by either method alone would be erroneous. Of course the actual amounts of two such optically active substances could only be determined after their separation. The want of harmony between the polarimetric and the reduction results would lead one to adopt some plan for the separation of the two bodies.

It is impossible, in this place, to describe the different forms of polarimeter. At present, instruments of the Laurent or Lippich type are employed where the zero point shows the whole field uniformly shaded, a slight rotation of the analysing Nicol to one side or other producing a corresponding darkening upon that side and brightening upon the other. The method of obtaining this hemisection of the field is

described in all books on experimental physics, or in such special works as Landolt's.¹

Any rotatory substance, other than the one whose percentage is being determined (glucose), must be removed. The one which is most commonly met with is lævo-rotatory protein. (For methods of removal, see p. 229.)

Consult such a book as Huppert's "Analyse des Harns" for methods of separating other optically active substances, as, for example, lævulose, paired glycuronic acids, and β -oxybutyric acid.

Process.

The urine must, if highly coloured, be precipitated with lead acetate (10 c.c. lead acetate (25 per cent.) for every 50 c.c. urine), filtered, precipitate washed, and filtrate measured.

In the first place, fix the zero point by directing the instrument to sodium flame, making the angle between the polariser and quartz plate as small as possible, and yet sufficiently large to permit of a reading, when the urine is being examined. This is done by rotating the polarising Nicol until the slightest movement of the analysing Nicol to one side darkens that side of the field, a corresponding one to the other darkening the other side.²

Focus exactly the dividing line before fixing the zero point.

Wash out tube with urine twice or thrice. Now pour through funnel into 1-dm. tube (or, better, a 2-dm. tube) urine (or decolorised filtrate), until, on removing the funnel, the fluid rises above the margin of the tube opening; slide on from one side the plane glass disc, avoiding pressing it down tightly, screw on the cap, and place tube in instrument between the two Nicols. See that no air bubbles are present. Focus again sharply the dividing line in the field. As the substance is probably dextro-rotatory (glucose), the half of the field to the observer's right will appear light, and that on the other side dark. Now rotate round analyser towards the observer's right until the previously bright half shows the same degree of dimness as the other (zero point), the least movement to either side now producing increased dimness of the corresponding side. Read off the angular rotation, or, if it be a saccharimeter, the percentage of sugar.

Knowing the angular rotation and the specific rotatory power of the substance (i.e. the angular rotation of a solution of the strength of 1 gm. in 1 c.c. examined in a layer 1 dm. long), the percentage can be calculated.

Of course, this means that it is known that only one optically active substance is present. This is usually glucose, and the calculation is made in the following way:—

The specific rotatory power is about $+52.5^\circ$, i.e. in a strength of 100 grms. of glucose per 100 c.c. solution, the solution being examined

¹ *Das optische Drehungsvermögen organischer Substanzen*, 2te Aufl., 1898.

² It is advisable to fix the zero point, using a 1-dm. or 2-dm. tube filled with distilled water between the polariser and analyser.

in a layer 1 dm. long. Thus a rotation of 1° would be produced by $\frac{100}{52.5}$ grms. of glucose per 100 c.c. solution. So that, having found the angular rotation of the solution of unknown percentage, the percentage is found on multiplying $\frac{100}{52.5}$ by the number of degrees (α) through which the analysing Nicol has been rotated.

That is, $p = \frac{100 \times \alpha}{[\alpha] \times l}$ where p = percentage in 100 c.c. solution; α = angular rotation; $[\alpha]$ = specific rotatory power; and l = length in decimetres of tube.

Solutions of different rotatory substances should be given out, their angular rotation determined, and the percentage calculated, the specific rotatory power being given.

Then a faintly coloured diabetic urine should be examined in the same way, also a highly coloured urine after treatment in the way previously described. It is also very instructive to determine polarimetrically the rotatory power of urine which reduces CuO before and after fermentation. The angular rotation in every case, when the urine has been filtered, must be corrected for the amount of urine actually taken.

6. By Fermentation.

This has been done in different ways—for example, from the difference in specific gravity before and after fermentation, or from the amount of alcohol or CO_2 formed. Unfortunately these methods are not very satisfactory.

7. By Johnson's Method.

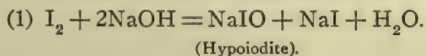
This is a colorimetric method, where a standard red-coloured solution is employed, the tint of which is about the same as that obtained when a known weight of glucose is boiled with KOH and picric acid in given proportions (red colour being due to picramic acid). This method, which is now obsolete, has been replaced by Benedict's colorimetric method ("Journal of Biol. Chem.," xxxiv. p. 203).

6. Acetone.

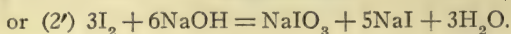
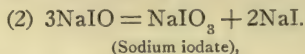
Estimation of total acetone (preformed and derived from aceto-acetic acid). Messinger and Huppert's method. (Embden and Schmitz's modification.)

PRINCIPLE OF THE METHOD.

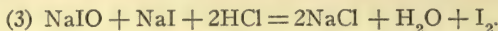
The method is based on the following reactions:—



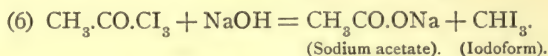
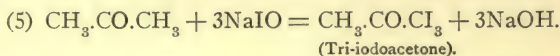
When the solution is allowed to stand for some time the following reaction occurs :—



On acidifying solutions (1) and (2), all the iodine is recovered, thus :—



Acetone is converted into iodoform by the action of sodium hypiodite, thus :—



Hence, three mols of iodine (3I_2) are required for the conversion of one mol of acetone (m.w. 58) into iodoform. 1 c.c. of N/10 iodine solution is therefore equivalent to 1 c.c. of M/60 acetone solution (i.e. 58/60 or 0.967 mgrm. of acetone).

REAGENTS REQUIRED.

(1) N/10 iodine solution (12.692 grms. per litre). Place 12.7 to 12.8 grms. of chemically pure, resublimed iodine in a stoppered weighing bottle. Determine the weight of weighing bottle and iodine to 1 mgrm. Transfer the iodine from the weighing bottle to a litre flask. Reweigh the bottle. The difference of the two weights (say 12.814 grms.) is the quantity of iodine taken. Dissolve about 25 or 26 grms. of pure potassium iodide (free from iodate) in about 250 c.c. of water (freshly distilled), and transfer the solution to the litre flask. Shake the solution gently from time to time until the iodine has completely dissolved. Then dilute the solution up to a litre with water at 15°. Measure from a burette 9.6 c.c. of distilled water into the litre flask, stopper the flask, shake the solution repeatedly, allow to stand for an hour, repeat the shaking, and transfer the N/10 iodine solution to a dry bottle with well-fitted glass stopper. The solution should be kept in the dark.

The reader is referred to works on volumetric analysis for methods of ensuring the accuracy of the N/10 iodine solution by titration against other standards.

(2) N/10 sodium thiosulphate (24.832 grms. of chemically pure crystalline sodium thiosulphate— $\text{Na}_2\text{S}_2\text{O}_3 + 5\text{H}_2\text{O}$ —per litre). A slightly stronger solution than this may be prepared, and its value determined by titration against the N/10 iodine solution. Other methods

of standardising the thiosulphate solution will be found in works on volumetric analysis.

(3) Starch solution. Dissolve 1 grm. of soluble starch in boiling water, cool, and make up to 100 c.c.

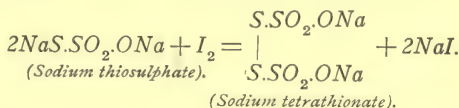
(4) 33 per cent. caustic soda.

(5) Dilute hydrochloric acid (1 vol. of concentrated acid mixed with three vols. water).

PROCEDURE.

Measure with a pipette 20 c.c. of urine into a 750 c.c. Erlenmeyer flask, add 150 c.c. of cold water, and 2 c.c. of 50 per cent. acetic acid. Connect the flask with an efficient condenser (Liebig or one of the more modern types). Place 150 c.c. of ice-cold water in 750 c.c. filtration flask, which serves as a receiver. Fit a rubber stopper through an opening in which a glass tube is inserted into the neck of this flask, and connect the tube with the condenser. Attach with rubber tubing a small Peligot U-tube containing a little ice-cold water to the side tube of the filtration flask. Allow a rapid stream of tap water to pass through the condenser. Heat the diluted urine to the boiling point, and maintain the boiling for 25 minutes. About 60 c.c. of fluid should distil over in this time. Remove the rubber stopper from the flask containing the urine, wash out the central tube of the condenser into the receiving flask, remove the receiving flask, and transfer the contents of the U-tube to the flask, washing out the tube thrice with water. Add 30 c.c. of (4), and from a burette with glass stopcock an accurately measured excess of N/10 iodine solution to the distillate. Mix the solution by gently rotating the flask, and allow the fluid to stand at room temperature for at least 5 minutes. A yellow precipitate of iodoform separates. Add one drop of (5). If the iodine be in excess, a brown colour appears where the acid meets the solution. If this does not occur, add more of the N/10 iodine, and allow the solution to stand for 5 minutes. If excess of iodine¹ is now present, acidify the solution with (5), cool thoroughly, and titrate the excess of iodine, set free with N/10 sodium thiosulphate until the yellow-brown colour of the iodine is nearly discharged. Then add a few drops of the starch solution, and gradually add N/10 sodium thiosulphate until the blue colour is just discharged.

The titration is based on the following reaction:—



CALCULATION.

Let x = number of c.c. of N/10 iodine solution added, and y = number of c.c. of N/10 sodium thiosulphate. Then $(x - y) \times 0.967$ mgrms. is the total quantity in milligrams of acetone in 20 c.c. of urine.

¹ The excess of iodine should be about 25 per cent. of the total amount added.

8. Ljundahl's Modification.

The foregoing method may also be carried out on a microchemical scale, if N/100 iodine and sodium thiosulphate solutions be used for the titration. One-tenth the quantity of urine (i.e. 2 c.c. measured with an Ostwald pipette) is required for each estimation, and the quantities of the other fluids added are reduced in the same proportion [e.g. 5 drops 25 per cent. acetic acid, 15 c.c. water, and 3 c.c. of (4)], with the exception of the water placed initially in the receiving flask, which should amount to 50 c.c.

A large number of other methods have been devised. Readers desiring fuller information are referred to the following papers:—Shaffer and Marriott, "J. Biol. Chem.," xvi. p. 276 (1914); Scott-Wilson, "J. of Physiol.," xlii. p. 444; Van Slyke, "J. Biol. Chem.," xxxii. p. 455.

Van Slyke and Marriott and Shaffer's methods have the great advantage of including β -hydroxybutyric acid in the determination.

7. Albumin and globulin.¹

There is a large number of methods which have been devised, some for exact analyses, others for clinical purposes. Three may be mentioned which have certain advantages over the others, for particular purposes at least.

[1. GRAVIMETRIC—REMOVAL BY COAGULATION.]

This is perhaps the most generally employed in scientific work, but numerous precautions require to be taken in order to render it accurate.

In many cases a large quantity of urine requires to be taken, from 100 c.c. upwards.² Take the reaction. If very slightly acid or alkaline, add one or two drops of acetic acid (1 in 2).³ In order to determine whether sufficient acid has been added, it is advisable to take a portion of the acidified urine in a test-tube, bring gradually to boiling point, filter, and test filtrate with acetic acid and potassium ferrocyanide. If precipitate appears, then add more acid to the large portion of urine, and repeat the testing of a small portion until finally that amount of acid has been added which is just sufficient to permit the coagulation of all (?) the albumin in the urine. Now heat the acidified solution to boiling point in large round-bottomed flask, fitted with upright condenser. It is advisable to use a paraffin bath. Filter now through a weighed folded filter paper (previously washed and dried). Wash with warm alcohol and then with ether, and dry filter paper in weighing bottle at 110° to 120° to constant weight (say one hour's drying).

The weight of the filter paper alone being known, and now its weight + the albumin, the amounts of coagulable protein in a known

¹ The methods employed for the accurate estimation of these bodies can only be satisfactorily carried out in an advanced laboratory class.

² The amount required will naturally vary with the probable amount of protein present. This can be gauged roughly by an ordinary test or an estimation in an Esbach tube.

³ Bang's method of coagulating protein may also be employed (see p. 226).

amount of urine, and in the twenty-four hours' excretion, are easily calculated.

If the urine be very poor in salts, it is advisable to add a few c.c. of a strong salt solution to the urine before boiling.

This method, if properly carried out, gives very good results, only is a rather tedious one. Instead of drying the coagulated protein, it may be incinerated by Kjeldahl's method, and the amount of nitrogen, on being multiplied by 6.3, gives the amount of protein.

[2. DIFFERENTIAL METHOD (BY KJELDAHL).]

This is the easiest method, when other nitrogen estimations are being made at the same time.

Estimate the total nitrogen in a measured amount of the urine, say 5 c.c., then carefully remove the albumin and globulin from 100 c.c. of the urine in the way just described, and estimate the nitrogen in a measured portion of the filtrate. As the amount of nitrogen present in the form of albumin in a small quantity of urine might be difficult to estimate accurately, it is advisable to take such a portion of the filtrate (say one quarter) as would enable one to detect a distinct difference in the percentage of nitrogen after the removal of the albumin. The fraction of the filtrate would require to be acidified slightly with H_2SO_4 , evaporated down to small bulk, and then incinerated by Kjeldahl's method in the usual way. The difference between these two results will give the amount of nitrogen removed in the form of coagulated protein. On multiplying this by 6.3, the amount of protein is determined.

This method is comparatively frequently employed in metabolic investigations when albuminuria has appeared.

3. ESBACK'S METHOD.

This is the method which is employed clinically. It only gives results which are of value when a series of estimations is being made in the urine of an individual from day to day, where density and general constituents do not vary markedly. When a certain quantity of a given solution of picric and citric acids is added to a measured quantity of urine of a certain density, the albumin is precipitated, and after a time forms a layer, the depth of which can be read off in a tube of a particular diameter. This tube can be graduated in percentages of albumin when the above conditions are fulfilled. In such estimations, great care has to be taken that the temperature is the same in every case, otherwise there will be great variations in the depth of the layer, even when there are none in the amount of albumin present.

The Esbach tube or albuminimeter has a mark, U (urine), 6 cms. from the bottom, 4 cms. above this an R (reagent), while from the bottom there are also lines of graduation stating the amount of protein in

grammes per litre, when the top of the layer coincides with a particular graduation.

Process.

If urine be not acid, acidify with acetic acid. Dilute urine until specific gravity is 1.006 to 1.008, noting the amount of dilution required. Pour urine now into a clean dry Esbach tube up to the mark U, and then Esbach's solution (picric acid 10 grms., citric acid 10 grms., in 1 litre of water) to mark R. Insert rubber stopper, mix by inverting the tube about a dozen times, and leave in a stand, in a place where the temperature does not vary much from day to day. After twenty-four hours, read off height of deposit. This gives the amount of albumin in grammes per litre. Correct for the dilution.

This method can only give results which are of value in clinical work, where much time cannot be bestowed on such an estimation.

The separate estimation of albumin and globulin in urine can be carried out by estimating the percentage of nitrogen before and after removal of globulin by saturation with sodium sulphate at room temperature, and, finally, after removal of albumin (see p. 74).

8. Albumoses (peptone).

In order to estimate these in the urine, it would be necessary to separate them from the urine, and then either weigh them or estimate their nitrogen percentage. Such estimations, however, would only be required in those rare cases in which a fairly large quantity of albumoses was present (see p. 228). Peptone is rarely, if ever, present in the urine.

The methods for the estimation of purine bases and a number of other urinary constituents (e.g. lactic acid and phenols) are not suitable for ordinary class work, and so are omitted. They are described in such books as Huppert's "Harnanalyse."

9. Method of recording the results of an analysis of urine.

The table from Folin given on p. 199 may be taken as a guide to the best method of stating the results. The date of collection of the urine, and the age and sex of the patient should also be given. It is also important to mention any drugs, which may have been taken by the patient.

If time permits, the freezing point of the urine, its P_H , and the phosphate ratio should also be determined. Any urinary sediment should be examined histologically and chemically. Finally, the urine should be tested for pathological constituents, and, if possible, their amounts determined.

Distribution of Nitrogen.

The determination of the total nitrogen or any one nitrogenous constituent such as urea has comparatively little value in throwing light

on the metabolism of the individual. On the other hand, valuable information regarding the metabolism of the patient may be gained if the values for urea-N, ammonia-N, creatinine-N, uric acid-N, and nitrogen of undetermined substances be each stated as percentages of the total nitrogen. One thus learns how the total nitrogen is distributed amongst the different nitrogenous constituents. The method of calculation is sufficiently indicated in the tables already referred to.

For fuller details the reader is referred to text-books of Physiology or works on Metabolism.

CHAPTER XX

BLOOD

1. Specific gravity.

The specific gravity of blood may best be determined by the method of Roy and Lloyd Jones. A series of solutions of sodium sulphate is prepared, ranging from a specific gravity of 1.035 to one of 1.070. The specific gravity of the blood of a healthy individual varies from 1.055 to 1.059.

1. *Place a series of the solutions in glass thimbles, arranging them in order of their specific gravities.*

2. *Take a small glass tube drawn out into a capillary tube, bent at right angles to the stem, and closed above by a caoutchouc cap. By means of it take up a drop of blood obtained from the finger.*

3. *At once introduce the point of the capillary tube into one of the solutions of sodium sulphate. Express a drop of blood by squeezing the caoutchouc cap, and note whether it sinks, floats, or remains suspended.*

4. *Repeat the process until a solution is found in which the drop of blood remains suspended.*

2. Determination of the gases of the blood by chemical methods.

(1) Oxygen capacity.

HALDANE'S FERRICYANIDE METHOD FOR DETERMINING THE OXYGEN CAPACITY OF THE BLOOD.¹

REAGENTS REQUIRED.

(a) Dilute ammonia (free from CO_2). Place some strong solution of ammonia (sp. gr. 0.88) in a bottle, add some powdered calcium oxide, shake up thoroughly, cork the bottle tightly, and allow the precipitate to settle. Add 4 parts of the clear supernatant fluid to 1 litre of boiled distilled water, and place the dilute solution of ammonia in a tightly corked bottle.

¹ May be demonstrated.

(b) Saturated solution of potassium ferricyanide.

Introduce into the bottle (a) (capacity 120 c.c.) by means of a pipette 20 c.c. of oxalated or defibrinated blood, previously saturated with oxygen, by shaking up with the air. In order to avoid admixture, with expired air, expel the last drops of blood from the pipette by closing the upper opening and warming the bulb with the hand. Then

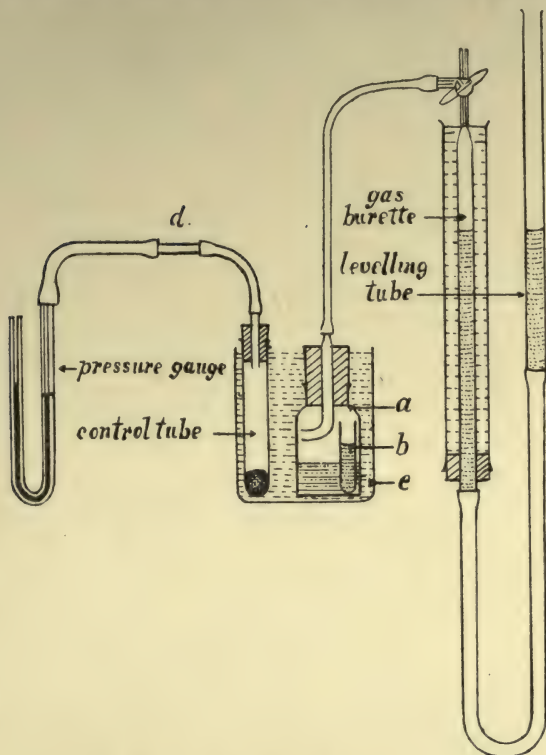


Fig. 13.—Haldane's Apparatus.

add 30 c.c. of dilute ammonia. The ammonia fixes the carbonic acid developed during the reaction, and the distilled water takes the blood. Mix the blood and dilute ammonia until the solution appears perfectly transparent when examined in a thin layer. Place 4 c.c. of a saturated aqueous solution of potassium ferricyanide in the small tube (b), which should slightly exceed in length the width of the bottle, and set the tube upright in (a). Push the rubber stopper into the neck of the bottle (a). The stopper is provided with a bent glass tube connected with the burette by means of thick-walled rubber

tubing of 1-mm. bore. The bottle (a) is then placed in a vessel containing water, having a temperature as nearly as possible that of the room. Open to the outside the three-way tap on the burette, and by raising the levelling tube adjust the water level in the burette so that it lies close to the top. Then fix the levelling tube in this position by means of the spring clamp, close the tap to the outside, and read off the water level in the burette, which is graduated to .05 c.c. Adjust the water in the pressure gauge to a definite mark, by sliding the rubber tubing backwards and forwards on the glass tube (d). After waiting until the reading in the burette has become constant, mix the diluted blood and solution of potassium ferricyanide by tilting the bottle (a). Surround the bottle with a towel to prevent its becoming heated by the warmth of the hand and continue shaking the bottle as long as gas is evolved. Then replace the bottle (a) in water. Add cold or warmed water to the water in (c), until the reading of the pressure gauge reaches its original level. As soon as the water level in the burette has become constant, carefully adjust the water in burette and levelling tube to the same level, and take the reading. The difference between the latter reading and that originally taken gives the volume of oxygen evolved. This volume may be reduced to that of the dry gas at 0° and 760 mm. in the usual way.

The following corrections are necessary in calculating the percentage of oxygen evolved:—A 20 c.c. pipette delivers only about 19.6 c.c. blood, and for this allowance must be made. Further, in accurate estimations, allowance must be made for the fact that the air in the bottle (a) has become richer in oxygen.

In making the latter correction, it may be assumed that the absorption co-efficients of oxygen and nitrogen in the 54 c.c. of fluid in the bottle are nearly the same as in water.

The volume of the dry gas (V_o) at 0° C. and 760 mm. may be obtained by using the following formula:—

$$V_o = \frac{v \times 273 \times (B - f)}{(273 + t)760}$$

v = observed volume of gas.

B = observed height of barometer in mm.

t = observed temperature.

f = vapour tension of water at temperature (t).

The value of (f) at different temperatures may be obtained from tables.

(2) Determination of the carbon dioxide content of blood.

REAGENT REQUIRED.

20 per cent. aqueous solution of tartaric acid.

After the determination of the oxygen capacity has been completed, remove the rubber stopper, and place a small tube containing 2.5 c.c. of the tartaric acid solution in the bottle (a). The further procedure

is the same as that required for the determination of the oxygen capacity, using tartaric acid solution instead of potassium ferricyanide.

Note 1. The CO_2 -content of blood as determined by the foregoing method will be found to be low owing to loss of CO_2 by the blood on exposure to atmospheric air. The following alternative method eliminates this source of error:—

Place about 40 c.c. of defibrinated blood in a 500 c.c. flask or separating funnel. After an ordinary inspiration, expire as completely as possible through a glass tube into the flask, thus displacing the atmospheric air with alveolar air. Close the flask with a rubber stopper, and rotate in a horizontal position for about 5 minutes, so as to expose as thin a layer of blood as possible to the alveolar air. In this way the gases of the blood are brought into equilibrium with alveolar air. The further procedure for the determination of the blood gases is the same as that already described, the separating funnel or flask being placed upright and 20 c.c. of blood withdrawn with a pipette.

Note 2. The technique of the method has been modified so as to render possible the determination of the blood gases in small samples of blood down to 0.1 c.c. The reader is referred to the following literature for details:—J. S. Haldane, "Journ. Physiol.," vol. xxii. p. 298, and vol. xxv. p. 295. J. Barcroft and J. S. Haldane, "Ibid.," vol. xxviii. p. 232. J. Barcroft, "The Respiratory Function of the Blood," pages 290 to 305. J. S. Haldane, "Journ. Path. and Bacter.," vol. xxiii. pp. 443 to 450.

Another form of apparatus for determining the CO_2 of blood plasma has been introduced by Van Slyke and Cullen ("Journ. Biol. Chem.," vol. xxx. p. 289 and p. 347).

3. Oxyhæmoglobin.

HALDANE'S MODIFICATION OF GOWERS' METHOD.

The apparatus consists of the following parts:—

1. The standard solution contained in a hermetically sealed tube (*b*) is a 1 per cent. solution of ox or sheep blood, saturated with coal gas. The oxygen capacity of the ox blood used is the same as that of the blood of normal adult males, namely, 18.5 per cent. The percentage of oxyhæmoglobin, corresponding to this oxygen capacity, is probably 13.8.
2. The tube (*c*) is graduated in percentages, and contains 2 c.c. when filled up to the mark 100.
3. The capillary tube (*a*), when filled up to the mark 20, contains 20 c.mm. (= .02 c.c.).
4. The bottle *A*,¹ with "teat" pipette stopper, contains distilled water for dilution of the blood.

¹ Not figured.

5. A shielded lancet is used for obtaining blood from the finger.
Method of using the instrument—

1. Place in the tube (c) sufficient water to dilute the blood as far as safely possible.

2. Obtain 20 c.mm. of blood from the finger by means of the capillary tube (a), and blow it into the water contained in the tube (c).

3. Before mixing the blood and water, pass down a piece of narrow glass tubing, connected with a gas tap, into the tube (c) nearly as far as the level of the fluid. The gas is turned on, and the air in the tube displaced. The gas delivery tube is withdrawn, while the current of gas is still maintained. The opening of (c) is then quickly closed

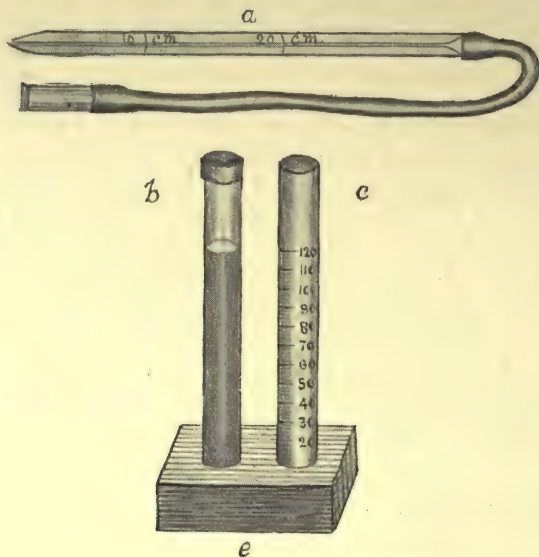


Fig. 14.—Haldane's (Gowers') Hæmoglobinometer.

with the finger. The liquid is then thoroughly mixed with the gas by inverting the tube about a dozen times, so as to ensure complete saturation of the blood pigment with CO.

4. Add water, drop by drop, from the test pipette A,¹ mixing the fluids well after each addition. Continue the addition of water until the tints of the fluids in the standard tube (b) and in the tube (c) appear equal. In comparing the tints, examine against the light from the sky, repeatedly transposing the tubes from side to side during the observation. Then read the percentage on tube (c). The results obtained should be within 1 per cent. of the mean of several observations.

This method of estimating the amount of the pigment in blood is

¹ Not figured.

described as one of the more generally applicable, and at the same time accurate methods.

Coloured standards of various descriptions have been used, but in all there is this one great drawback—that one is entirely in the hands of the instrument-maker as regards accuracy of the standard. The most commonly employed hæmoglobinometers are those of Gowers, Oliver, Fleischl, and a most useful modification of the last-mentioned apparatus, namely, that of Miescher.

It is much better, however, that each student should, as far as possible, gain experience in the use of one accurate and yet easily applied method, than that he should have only a theoretical knowledge of a number of different forms of apparatus. The spectro-photometric method does not, of course, come within the scope of such an elementary text-book as this.

4. Blood Gases.

EXTRACTION OF THE BLOOD GASES.

The gases of the blood can be extracted by means of Leonard Hill's gas pump. The method of using the instrument may be shown as a demonstration (see "Journ. Physiol.," vol. xvii. p. 353). A more accurate method has been devised by J. Barcroft ("J. Physiology," xxxix. p. 433).

5. Quantitative analysis of blood.

(1) Reaction (see pp. 272-273).

(2) Total nitrogen.

Place about 2 c.c. of defibrinated blood in a small dry beaker. If blood drawn from a vein be used, first place about .01 gm. of finely powdered pure potassium oxalate in the beaker, and mix the blood and salt by rotating the beaker. Weigh accurately the beaker and its contents. Let weight be x g. Pour the blood into a Kjeldahl flask, and immediately reweigh the beaker (y g.). Heat the blood with sulphuric acid in the way already described (pp. 312-316). Mercury may be used with advantage instead of copper sulphate to hasten the oxidation. Let k be the quantity of nitrogen in grammes found by Kjeldahl's method, then $100 k/x - y$ is the percentage by weight or quantity of nitrogen in 100 grms. of blood.

(3) Iron (see p. 254).

(4) Micro-chemical analysis of blood.¹

A large number of microchemical methods have been devised for the

¹ For descriptions of many of the methods, and references to further literature, the reader may consult J. Bang (1916), "Mikromethoden zur Bestimmung einiger Blutbestandtheile," Wiesbaden, Bergmann. S. W. Cole (1920), "Text-Book," pp. 250 to 264. O. Folin (1919), "Laboratory Manual of Biological Chemistry," pp. 179 to 213, New York, Appleton. P. B. Hawk (1918), "Practical Physiological Chemistry," pp. 274 to 301, Blakiston, America, and Churchill, London. H. Maclean, "On the Estimation of Sugar in Blood," "Bioch. J.," xiii. p. 135.

estimation of the nitrogenous non-protein constituents of human blood (e.g. non-protein nitrogen, urea, ammonia, amino-acids, creatine, creatinine, and uric acid), as well as the following non-nitrogenous constituents:—glucose, cholesterol, fats, and, in pathological cases, acetone, aceto-acetic, and β -hydroxybutyric acids.

Only one of these methods, namely, the urease method for urea, is described, since the technique is relatively simple. Scrupulous accuracy in all measurements of weight or volume is obviously even more essential in micro-chemical than in macro-chemical estimations, since errors of observation are greatly magnified in calculating the experimental results as percentages.

Urea Estimation (D. Van Slyke and Cullen's modification of Marshall's method).

METHOD.

The apparatus required is the same as that for Van Slyke and Cullen's method for the estimation of urea in urine, and the technique is essentially the same.

Place 5 c.c. of 0.6 per cent. potassium citrate solution (to prevent clotting) in the boiling tube B. Measure 3 c.c. of whole blood (plasma or serum) into the tube from a pipette graduated to contain 3 c.c., washing out the pipette as thoroughly as possible with the citrate solution. Add 2 drops of Folin's buffer solution (6.9 grms. monosodium phosphate and 17.9 grms. crystalline disodium phosphate per 100 c.c.), 0.5 c.c. urease solution (or a ground urease tablet), and 3 drops caprylic alcohol to prevent frothing. Fit up the apparatus as described on p. 321, placing 15 c.c. of N/100 sulphuric acid in the absorption tube and immersing B in water at 45°. Draw a slow current of air through the apparatus for at least 15 minutes, and a rapid current for 1 minute. Remove the water bath, raise the stopper of tube B, and add about 15 c.c. of a saturated solution of potassium carbonate, insert the stopper in B, and mix the fluids by shaking. Again immerse B in a water bath at 45°, and draw a slow current of air through the apparatus for 2 or 3 minutes, and a rapid current for about 15 minutes. Stop the air current, remove the absorption tube, and titrate the excess of acid with N/100 NaOH (free from carbonate), using methyl-red as indicator.

CALCULATION.

Let x = number of c.c. of N/100 NaOH required, then $(15 - x) \times .0003 \times 33.3$ or $(15 - x) \times .01$ is the quantity of urea in grammes in 100 c.c. of blood, and this value multiplied by 0.467 gives the quantity of urea-nitrogen in 100 c.c. of blood.

Note.—The preformed ammonia-nitrogen of the blood is calculated as urea in this method. The quantity of preformed ammonia in normal blood is so small that the error involved is usually negligible.

The method described on pp. 322 and 323 (5) may also be applied to the estimation of urea in blood.

CHAPTER XXI

MILK¹

THE main constituents of milk may be quantitatively estimated in the following way:—

1. Specific gravity.

The specific gravity is usually determined with an accurate milk hydrometer graduated from 1.024 to 1.038, the distance between each division being 7.5 mm.

PROCEDURE.

Place well-mixed milk in a glass cylinder, removing any foam on the surface with filter paper. Gradually lower the dry hydrometer into the milk until the surface of the fluid stands at about 1.030 on the scale, release the hydrometer, and, when it has become stationary, read the level of the surface of the milk on the scale, keeping the eye at the same level as the surface of the milk. Add 0.1 of a degree to the reading as a correction for the raising of the level of the surface of the milk round the stem of the hydrometer, e.g. sp. gr. reading 1.0315 (corrected reading 1.0316). If the temperature of the milk be above or below 15°, correct the reading by means of Müller's table (see works on milk analysis).

2. Reaction.

(1) See page 272 for "indicator method" of determining the reaction of whey from clotted milk, and pp. 273 to 280 for the electro-metric method.

(2) Titratable Acidity (Thörner's method).

Mix 10 c.c. of milk with 20 c.c. of distilled water, add 5 drops of 5 per cent. alcoholic solution of phenolphthalein, and titrate with N/10 NaOH until a faint, permanent red colour is obtained. Multiply the number of c.c. of N/10 alkali added by 10. The result gives the degree of acidity of 100 c.c. of milk. The average acidity of fresh milk is 16 to 18 degrees. When the acidity is over 25 degrees, the milk coagulates on boiling.

¹ The quantitative analysis of milk can only be carried out satisfactorily in an advanced class.

Multiply the number of c.c. of N/10 alkali added by .009. The result gives a rough estimate of the amount of lactic acid in grammes in 10 c.c. of milk.

An alternative method which is less convenient for most laboratories is that devised by Soxhlet and Henkel. In this method, 100 c.c. of undiluted milk plus 4 c.c. 2 per cent. phenolphthalein solution are titrated with N/4 NaOH (see p. 188).

3. Total Solids.

Weigh out from a small beaker 2 to 3 grms. of milk into a small porcelain basin, which has previously been heated, cooled, and weighed. Dry the basin and its contents at 100° for three hours, cool in exsiccator, and weigh. Again dry at 100° for an hour, cool, and weigh. If the two weights are the same within 0.2 to 0.3 mgrms., the drying is complete. If not, the basin and contents must be again dried until the weight is constant. Deduct the weight of the basin from that of basin plus dried solids. The result gives the amount of dried solids in the quantity of milk taken for analysis. The percentage of dried solids can then readily be calculated.

4. Ash.

Place about 10 grms. of milk in a weighed platinum or porcelain basin, evaporate to dryness on the water bath, and incinerate in the way described on page 256.

5. Total nitrogen.

Take 10 c.c. of the fresh milk in a Kjeldahl flask, incinerate with H_2SO_4 in the usual way, and distil off the ammonia from the fluid after making alkaline (see pp. 312 to 316). Calculate in terms of nitrogen.

6. Caseinogen, lactalbumin, lactoglobulin.

As Hoppe-Seyler's method can be comparatively rapidly carried out, it will be described here, although certain other methods are more reliable, if the total albuminous nitrogen is to be estimated.

(a) CASEINOGEN.

Take 25 c.c. milk in a glass cylinder, dilute with water up to 500 c.c., acidify with a few drops of acetic acid, and shake thoroughly. Examine the surface layer, with the cylinder inclined, to see whether flocculi have begun to separate out. If there be no appearance of a precipitate, add a few more drops of the acid, shake thoroughly, and again examine. Whenever the fluid shows a distinct flocculent precipitate, no more acid requires to be added. Now, pass a stream of CO_2 through the fluid for about half an hour, and then allow the

precipitate to settle for at least two hours. Siphon off supernatant fluid and filter the remainder through folded filter paper. If necessary, also run the supernatant fluid through the same paper. Wash the precipitate once or twice with water containing approximately the same amount of acetic acid as the solution out of which the caseinogen separated. Make up filtrate and washings to a definite volume, and take a measured portion of this (say one quarter) for nitrogen estimation. Run this fraction of the filtrate into a Kjeldahl flask, add a few drops of concentrated H_2SO_4 , and evaporate down to small bulk over the open flame. Then add 15 c.c. of the concentrated H_2SO_4 , a few crystals of $CuSO_4$, and 5 to 10 grms. of K_2SO_4 . Incinerate. The nitrogen is then estimated in the usual way.

This gives the nitrogen in a fraction of the total filtrate (freed from caseinogen) from 25 c.c. milk. If the amount of nitrogen in the fresh milk be known, and also that of the milk from which the caseinogen has been removed, the difference will give the nitrogen in the form of caseinogen, and on multiplying this by 6.37 the amount of caseinogen itself is arrived at.

(b) LACTALBUMIN AND LACTOGLOBULIN.

Take the rest of the caseinogen-free filtrate (i.e. three-quarters), add one quarter of saturated $NaCl$ and bring to boiling point. A drop or two of dilute acetic may then be added if the coagulation be not well marked. Filter through folded paper while fluid is still hot, wash precipitate with hot water, and make up total filtrate, plus washings, to a definite volume. Estimate in a fraction of this (say one-half) the nitrogen.

This represents the nitrogen of the milk from which caseinogen, lactalbumin, and lactoglobulin have been removed, and knowing the nitrogen percentage of the milk without caseinogen, the percentage of the other proteins is at once obtained (their nitrogen $\times 6.37$ = amount of lactalbumin and lactoglobulin).

7. Fats.

These may be estimated in the following way:—

Reagent. Ether.

Purify 500 c.c. of ether by allowing it to stand in contact with 40 grms. of powdered caustic soda for 24 hours, and distil off the ether on the water bath.

PROCEDURE.

Pin the ends of a fat-free strip of filter paper ($22 \times 2\frac{1}{2}$ inches) to two pieces of wood, and fix the ends of the latter in clamps so as to keep the strip of paper loosely stretched horizontally. Then allow 5 c.c. of milk to drop slowly from a burette on the paper in such a way

as to distribute the milk as evenly as possible over the surface. Heat the paper very gently with a small Bunsen burner placed some

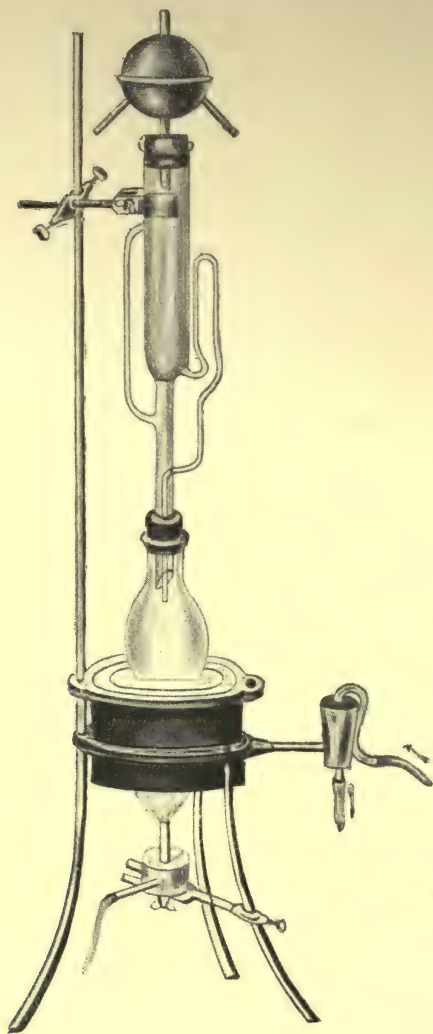


Fig. 15.—Soxhlet's Extraction Apparatus.

distance below its lower surface. When the paper is practically dry, roll it up into a cylinder, fix in position with a thread, place in a cartridge paper, and dry for an hour in a beaker placed in a hot

water oven. Now place the cartridge paper and contents in a Soxhlet extractor, and connect with a vertical condenser above and an ether flask below (see fig. 15). See that all connections are secure, then lower the flask upon the sand bath, maintained at suitable temperature, to keep the ether just boiling. The ether vapour condenses in the cooled upper tube, drops upon the dried milk, and, when the ether rises above the level of the siphon tube which connects the Soxhlet tube with the ether flask below, the fluid is siphoned over into the latter. After about two to three hours' extraction,¹ extinguish the small flame, remove the flask, distil off the ether, and dry the residue in a vacuum exsiccator. Then, after one hour, weigh the stoppered flask, and if the weight of this flask when empty be known, the weight of the fat of 5 c.c. milk is obtained. State the amount in percentage.

8. Lactose.

May be estimated in a measured amount of milk which has had its caseinogen, lactalbumin, and lactoglobulin removed by the method just described. The filtrate from the protein precipitate should be neutralised before titration.

(1) Volumetric method.

A burette is filled with the filtrate so obtained, and the fluid is run into a measured quantity of boiling Fehling's solution until reduction is just completed. Each c.c. of the Fehling's solution = 6.7 mgrms. lactose. Thus, if one knew the volume of the total filtrate obtained from a measured quantity of milk, the percentage of lactose is easily obtained.

The lactose in milk may also be determined by Folin and MacEllroy's method ("Folin's Manual," p. 139, also Folin and Denis, "J. of Biol. Chem.," xxxiii. p. 521). The great advantage of the latter method is that it does not involve removal of the proteins, and the procedure is therefore greatly simplified.

(2) Polarimetric method.

Place 80 c.c. of milk in an accurately graduated 100 c.c. stoppered cylinder, make up to 100 c.c. with 20 c.c. of 10 per cent. aqueous solution of trichloroacetic acid, mix thoroughly, allow to stand for 5 minutes with frequent shaking, and filter through dry filter paper. The filtrate which is usually slightly turbid, must be refiltered until it has become perfectly clear. Place the clear filtrate in a 2 dm. tube and examine with the polarimeter in the way described on pages 20 and 21.

EXAMPLE.

$$+52.53^{\circ} = \frac{100 \times 3.98^{\circ}}{2c} \therefore c = 3.79 \text{ and } \frac{3.79 \times 5}{4} = 4.74 \text{ per cent. (by volume)}$$

of lactose.

¹ It is often necessary to extract for a longer period, otherwise some fat may not be extracted.

CHAPTER XXII

GASTRIC JUICE, ETC.

I. Estimation of acidity.

(1) Reaction.

See pp. 269 and 280 for "the indicator" and electro-metric methods of determining the pH values of the gastric contents.

(2) Total titratable acidity.

This may be done simply by titrating 10 c.c. of the filtered gastric contents¹ with $\frac{N}{10}$ NaOH, using phenolphthalein as an indicator (3 drops of 1 per cent. alcoholic solution). This will give approximately the total acidity, but the end point is not well marked. The decinormal NaOH is added until there is a permanent faint red tint (pH 8.4). The result may be stated in terms of HCl or in terms of c.c. NaOH used up. This titration gives the total acidity of the gastric contents due to free HCl, loosely combined HCl (*i.e.* combined as acid metaprotein, and with proteoses and peptone), organic acids (mainly lactic acid), and acid salts.

2. Free hydrochloric acid.

This may be approximately determined by Töpfer's method.

(a) *Add 4 drops of 0.5 per cent. alcoholic solution of*

¹ Some observers prefer to titrate the unfiltered gastric contents, or the supernatant fluid obtained after centrifugation. When the gastric contents are filtered, part of the HCl combined with protein is retained by the filter paper. If the gastric contents be well shaken before filtering the loss of acid is small (Christiansen), and the colour changes of indicators are more easily observed in a clear filtrate.

dimethylaminoazobenzene to 10 c.c. of the filtered gastric contents, and titrate with N/10 NaOH until the red colour of the solution becomes yellow.

Although this method can readily be proved to be inaccurate by applying it to the analysis of solutions containing known quantities of HCl, and lactic acids, it has the advantage of simplicity, and gives valuable comparative results in clinical work.

CALCULATION.

The results of 1 (2) and 2 (a) are often stated in terms of HCl per 100 c.c. gastric contents. Let x be the number of c.c. of N/10 alkali required for titration 1 (2), y be the corresponding value for titration 2 (a), then $.0365x$ per cent. HCl represents the total acidity of the gastric contents in terms of HCl, and $.0365y$ gives the percentage of free HCl in the gastric contents.

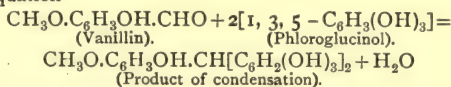
(b) The following method is more accurate than (a), but less convenient :—

Heat a flat porcelain dish (or porcelain basin) on the water bath, and place on it a series of drops of Günzburg's reagent (or Boas' reagent, see pp. 106 to 107).¹

Measure with a pipette two 10 c.c. portions of the filtered gastric contents into small Erlenmeyer flasks labelled A and B. With a glass rod withdraw a drop from A, and bring into contact with one of the yellowish spots left on the porcelain plate after evaporation of a drop of Günzburg's reagent. If a carmine red colour develops as the solution dries, free hydrochloric acid is present²

¹ The reagent (p. 106) should be stored in a brown or covered bottle and kept in the dark. When fresh it is nearly colourless. Solutions which have become distinctly brown should be rejected. The Steensma-Günzburg reagent, which is also used, contains methyl in place of ethyl alcohol. Boas' reagent is more stable but less delicate than Günzburg's.

² The chemical reaction, on which this colour test depends, may be represented by the equation—



The product of condensation forms a red coloured compound with traces of HCl.

(see (c) if result negative). Add to A 0.5 c.c. N/10 NaOH from a burette, mix thoroughly, withdraw a drop and test with Günzburg's reagent. If the reaction be positive, add another 0.5 c.c. N/10 NaOH, and again test a drop with Günzburg's reagent. Continue adding N/10 NaOH (0.5 c.c. at a time), testing a drop with Günzburg's reagent after each addition until a negative result is obtained. Let us assume that a positive result was obtained after 2.5 c.c. of N/10 NaOH had been added and a negative result after 3 c.c.

Add 2.6 c.c. of N/10 NaOH to the contents of B, mix thoroughly, and test a drop with Günzburg's reagent. Continue adding 0.1 c.c. N/10 NaOH at a time, testing with Günzburg's reagent after each addition, until a negative result is obtained. Let us assume that a positive result was obtained after 2.8 c.c. N/10 NaOH had been added, and a negative result after the addition of 2.9 c.c. N/10 NaOH. The free HCl of 100 c.c. of the gastric contents is therefore 29 c.c. N/10 HCl, or the percentage by volume of HCl is $.00365 \times 29$ or 0.106 per cent. The former method of stating the result is the simpler, and the one most frequently adopted.

(c) "Hydrochloric acid deficit."

If the gastric contents give a negative result with Günzburg's reagent, free hydrochloric acid is absent.

The deficit may be determined in the following way:— Measure 10 c.c. of the filtered gastric contents into a small flask and titrate with N/10 HCl until a positive result with Günzburg's reagent is obtained. Assume that 0.7 c.c. N/10 HCl are required, then the HCl deficit per 100 c.c. gastric contents is 7.

A number of dibasic organic acids give a similar reaction; but all monobasic and hydroxy-monobasic organic acids give a negative result. According to Christiansen the colour change is not directly dependent on the initial pH of the solution, but only occurs when free acids are present, which, on evaporation, yield residues having a high degree of acidity, approximately equivalent to N/1 HCl.

3. Estimation of HCl ("free and loosely combined").

(a) The method that is most commonly used is that of *Mörner and Sjöqvist*.

Take approximately .5 gm. pure chlorine-free BaCO_3 in a platinum crucible. The crucible is placed upon the hot water bath, and 10 c.c. of the filtered gastric juice are added in small portions, the contents being gradually dried. As the evaporation goes on, the acid fluid is run in slowly until the 10 c.c. have been added. The HCl is fixed as BaCl_2 , the lactic acid as barium lactate. The dried mixture is now incinerated over a small flame until the mass of the carbon has disappeared. The lactate is now in the form of BaCO_3 , while the BaCl_2 has resisted the incineration. Extract the cold residue with small quantities of hot water until the washings no longer give a chlorine reaction. The collected extracts are placed in a beaker which is immersed in a hot water bath, and dilute H_2SO_4 is added until all the soluble barium salt is precipitated as insoluble BaSO_4 . Excess of the dilute acid is added. The BaSO_4 precipitate is treated in the way described on p. 306. On multiplying the weight of the BaSO_4 by .3123, the amount of HCl in 10 c.c. of the gastric contents is obtained.

This method gives the total HCl (free and loosely combined or "physiologically active"). Ammonium chloride is calculated as HCl in the result obtained by Mörner and Sjöqvist's method. The quantity of ammonia is usually so small as to be negligible. It may be determined by the methods employed for the estimation of preformed ammonia in urine (pp. 310-312, and p. 322).

(b) J. Christiansen has found that if sensitive litmus paper be used as an indicator in the titration of the gastric contents with N/10 NaOH, the titration values are usually nearly identical with the results obtained by Mörner and Sjöqvist's method. The sensitive litmus paper is prepared in the way described on p. 287.

PROCEDURE.

Measure two 10 c.c. portions of the filtered gastric

contents into flasks labelled *A* and *B*. Place a drop of the fluid from *A* on a slip of the litmus paper. If a distinct red colour be produced, the contents are acid. Add 0.5 c.c. *N*/10 NaOH, mix thoroughly, and test with litmus paper. Continue adding *N*/10 NaOH (0.5 c.c. at a time) until a blue spot is obtained, when a drop is placed on the litmus paper. Let us assume that 10 c.c. of the gastric contents gave an acid reaction with litmus after the addition of 5.5 c.c. *N*/10 NaOH and an alkaline one after 6 c.c. *N*/10 NaOH. Repeat the titration with *B*, starting with the addition of 5.6 c.c. *N*/10 NaOH, and adding 0.1 c.c. *N*/10 NaOH at a time, until a drop of the solution gives the same colour with the litmus paper as a drop of the second phosphate mixture (see p. 287). Let the result be 5.7.

Tabulate the results of the above experiments in the following way:—

100 c.c. of the gastric contents contained		
Free HCl (Günzburg).	“ Physiologically active or free and loosely combined HCl ” (Litmus).	Total acidity in terms of <i>N</i> /10 acid.
29 c.c. ($\frac{N}{10}$ acid.)	57 c.c. (<i>N</i> /10 acid.)	[65 c.c.]

For fuller details regarding these methods the reader is referred to J. Christiansen, “*Bioch. Zeitschr.*,” xlv. pp. 24 to 93.

NOTE.—Mixtures containing known amounts of HCl (*N*/20 to *N*/40) proteoses and peptone (1 per cent.) may be used for class work.

4. Estimation of total chlorides of the gastric contents (*i.e.* fixed and volatile chlorides).—Method of Prout-Winter modified by Reissner and others.

Place in a porcelain crucible sufficient *N*/10 NaOH (free from chlorides) to neutralise 20 c.c. of the gastric contents, using the result of titration 1 (2) as a guide.

Add from a burette 5 c.c. of the filtered gastric contents, mix well, and evaporate to small bulk on the water bath. Add an additional quantity of the filtered gastric contents, again evaporate, and repeat the addition of the filtered gastric contents until 20 c.c. have been added. Evaporate to complete dryness on the water bath, and finally incinerate in the way described on p. 256. Allow the crucible to cool, thoroughly extract the residue with a few c.c. of hot water, cool, and filter the extract into a 100 c.c. flask. Extract the residue again with a few c.c. of dilute nitric acid (1 in 20), and filter the extract into the flask. Repeat the extraction and filtration until the total filtrate is about 60 c.c., add 20 c.c. N/10 silver nitrate solution, make up to 100 c.c. with distilled water, and mix thoroughly. The chlorides are then determined by Volhard's method (for principle of method see pp. 299 - 300).

REAGENTS REQUIRED.

(1) N/10 silver nitrate solution (16.99 grms. per litre). Weigh approximately 17 grms. (17.1 - 17.2 grms.) of pure fused silver nitrate, place in a weighing bottle, and weigh accurately the bottle and its contents. Transfer the salt to a litre flask, ascertain the amount of silver nitrate by reweighing the bottle, dissolve in water, and make up to a litre. Add from a burette the calculated volume of water required to bring the solution to N/10 strength, and mix thoroughly by repeated shaking.

(2) N/10 ammonium thiocyanate (7.612 grms. per litre) or N/10 potassium thiocyanate (9.718 grms. per litre). Dissolve about 10 grms. of pure ammonium thiocyanate or 13 grms. of pure potassium thiocyanate in water, make up to about 1200 c.c. with distilled water, and mix thoroughly. Standardise this solution in the following way:—Place 20 c.c. of N/10 AgNO_3 in an Erlenmeyer flask, add 5 c.c. of 3 per cent. ferric ammonium sulphate, and 4 c.c. 30 per cent. pure nitric acid. Then run in

gradually from a burette the thiocyanate solution until a faint permanent red colour is obtained.

If x be the number of c.c. of thiocyanate required, then $\frac{1000(20 - x)}{x}$ c.c. of water must be added to 1 litre of the stock solution in order to bring it to $N/10$ strength. If the latter value exceed the capacity of the neck of the litre flask, dilute the solution further in a measuring cylinder with the amount of water required to make it slightly stronger than $N/10$, and again standardise by titration against 20 c.c. of $N/10$ AgNO_3 . Then pour the solution into a litre flask accurately up to the mark, add from a burette the quantity of water required to bring it to $N/10$ strength, and mix thoroughly by repeated shaking.

METHOD.

Filter off the contents of the 100 c.c. flask from the precipitate of silver chloride through dry filter paper into a dry flask. Measure 75 c.c. of the filtrate with a 50 and a 25 c.c. pipette into another flask, add 5 c.c. of iron alum solution and 3 c.c. pure 30 per cent. nitric acid, and titrate the excess of silver nitrate with $N/10$ thiocyanate. Let x c.c. be the quantity of thiocyanate required, then $\frac{(15 - x) \times .00365 \times 5 \times 4}{3}$ is the percentage (by volume) of chlorides in terms of hydrochloric acid.

NOTE.—15 replaces 20 since only $\frac{3}{4}$ ths of the total solution were titrated, and the result is multiplied by $\frac{4}{3}$ for the same reason; and by 5 to convert to percentage, 20 c.c. of the gastric contents being taken for analysis.

5. Non-volatile or fixed chlorides.

Repeat the above method; but omit the initial addition of $N/10$ NaOH . The result gives the percentage of fixed chlorides stated in terms of HCl . Deduct the result of 5 from that of 4, the difference gives the percentage of physiologically active or free and loosely combined hydro-

chloric together with ammonium chloride, if the latter be present.

The reader is referred to a paper by G. Graham (*Quarterly Journal of Medicine*, iv. p. 315) for fuller details regarding methods 4 and 5.

6. The gastric contents should also be qualitatively examined for the products of amylolytic and proteolytic digestion (p. 102 and pp. 113 to 115) and for lactic acid (pp. 107 and 108).

7. Determination of amino-nitrogen set free (pp. 285 and 286).

8. Pathological constituents.

Volatile fatty acids (acetic and butyric), bile (see pp. 146, 147, and 232 for tests), blood (p. 160 (c) and p. 231 (b)), tryptophane (p. 121 (c)), or indole (pp. 130 and 131) may be present.

NOTES

Detection of butyric acid.—*Extract 10 c.c. of the gastric contents with 20 c.c. of ether in a separating funnel, separate the ethereal solution, and evaporate on the hot water bath (extinguishing flames in the vicinity). Dissolve a little anhydrous calcium chloride in the aqueous residue. If butyric acid be present oily droplets separate, and the characteristic odour of the acid can be readily recognised.*

The detection of blood has no pathological significance unless a blood free diet has been given, and any possibility of injury of the gastric mucosa during the removal of the gastric contents can be excluded.

Traces of bile and tryptophane may occur in the gastric contents under normal conditions.

9. For the composition of "tests meals," and methods of obtaining gastric contents, the reader is referred to textbooks on Clinical Diagnosis.

For a full account of "the fractional method of gastric analysis" the reader may consult P. Hawk's "Practical Physiological Chemistry," pp. 151 to 166.

Serous fluids, etc.

A quantitative analysis of the constituents of physiological and pathological serous fluids can only be carried out in cases where the student can devote much time to laboratory work, and hence is not suitable for ordinary class work.

This is true also of analysis of muscles, of the different organs, etc. For this reason, no description of methods suitable for these analyses can be given here.

CHAPTER XXIII

SOME APPLICATIONS OF THE POLARIMETER IN BIOLOGICAL CHEMISTRY

Optical construction of the polarimeter.¹

THE polarimeter is an instrument used for measuring the angular rotation of polarised light produced by optically active substances.

The simplest form of the instrument consists of two Nicol prisms, a polariser and an analyser, mounted on the same axis. A glass tube, containing the pure, optically inactive solvent, usually water, is closed at both ends with plane glass plates, and interposed between the two Nicols.

All the observations are carried out in a dark room. A source of natural homogeneous light, usually sodium chloride or bromide heated in a bunsen flame, is placed in the same optical axis as the polarimeter. The rays of natural homogeneous light from the source, which vibrate in all directions perpendicular to the line of propagation, traverse the polarising Nicol, and emerge from it as plane polarised light. The rays of polarised light then pass successively through the water in the glass tube and the analysing Nicol, and are viewed by the observer through a telescope mounted in the same metal tube as the analyser. If the analysing Nicol be rotated, then the circular field will be twice completely dark, and twice bright during a complete revolution, the positions of maximal brightness and complete darkness being separated by an angle of 90° . If the Nicols be "crossed," or, in other words, the analyser be so set that the field appears completely dark, and a glass tube containing cane sugar be placed between the polariser and analyser, the circular field is found to be no longer completely dark. If the analyser be now rotated clockwise ("dextrorotation") through a small measured angle, the field will again become dark. This angle gives the amount and direction of rotation of the plane of the polarised light by the optically active substance, cane sugar. On the other hand, when the analyser has to be rotated "anticlockwise" through an angle less than 90° in order to obtain complete extinction of the light, the optically active substance

¹ The following description is confined to the most essential practical details, and the reader is, therefore, referred to works on Optics (such as R. A. Houstoun's "Treatise on Light," pp. 186 to 203, and 212 to 219. London: Longmans), and on stereochemistry (such as A. W. Stewart's "Stereochemistry") for the theory of the polarimeter, and the nature of optical activity.

(e.g. fructose) is said to be lævorotatory. Obviously, a position of complete darkness could also be obtained by rotating the analyser in either direction since there are two positions of darkness 180° apart. That direction is chosen which requires rotation of the analyser through an angle less than 90° in order to obtain extinction of the light.

The amount of rotation depends on the following factors:—

(1) The nature of the substance. The direction of rotation is also dependent on (1).

(2) The number of optically active molecules traversed by the light, or, in other words, on the concentration of the optically active solute and the length of the column of solution.

(3) The temperature.

(4) The wave length of the light, being approximately proportional to its inverse square. White light, if used, as a source, therefore undergoes rotatory dispersion. When the analyser is rotated the field never becomes completely dark, but a series of colours are seen. The illumination of the field is least for a bluish-red tint which is taken as the zero. More accurate measurements are obtained when homogeneous light is used as the source.

The optical activity of a solute is defined by its specific rotation (see pp. 20, 21, and 330). The molecular rotation, indicated by the symbol $[\alpha]$, is equal to the specific rotation multiplied by the molecular weight (m) and divided by 100.

The simple type of polarimeter just described is very inaccurate, since it is difficult to set the analyser so as to obtain a completely black field. The field appears black over a considerable angular range. The construction of more accurate instruments is based on "the half shadow" principle.

Figure 16 represents the optical arrangements of the Lippich polarimeter. The polariser consists of two Nicols, one large Nicol, P, which can be rotated about its long axis by means of a lever through a small angle (about 5°), thus altering its position relatively to the small Nicol, Q, which covers half the field, and forming the half shadow angle. Beyond the Nicols lies the aperture B. C is another aperture and A the analysing Nicol. The latter is contained in a tube, to which a graduated disc is fixed. When the analyser is rotated round the axis of the instrument the disc rotates with it past a fixed vernier. The difference between two readings on this disc gives the angle through which the

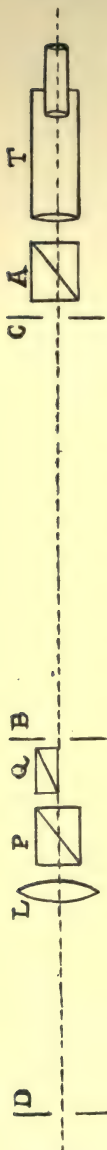


Fig. 16.

OPTICAL CONSTRUCTION OF THE POLARIMETER 361

analyser has been rotated. The observation tube containing the solution to be examined is placed between B and C.

Monochromatic light (*e.g.* yellow light from a sodium flame) is obtained by placing fused sodium chloride or bromide in a platinum ring inserted into a bunsen flame. The source of light should be placed in alignment with the axis of the instrument, and at such a distance from the aperture D that, when the observation tube is inserted, an inverted image of the flame falls on a piece of white paper placed before the aperture C. This adjustment may be facilitated by bringing a platinum wire into the bunsen flame, and altering the distance of the bunsen from the polarimeter until a distinct image of the wire is obtained on the paper.

L is a lens and T a small telescope which is focussed on the edge of Q by adjusting the position of the eyepiece. A cell containing potassium bichromate is placed between the sodium light and the lens L in order to absorb all but the yellow rays.

On looking into the telescope, the observer sees a circular yellow field usually divided into two halves of unequal brightness separated by a line, the image of the edge of Q. One half of the field is illuminated by plane polarised light, which has emerged from P, and the other half by light, which has passed through P and Q. The light in the two halves of the field is polarised in planes M and N at a small angle, 2α (fig. 17), to one another. If the analysing Nicol is so rotated that its principal plane is perpendicular to the plane of polarisation of half the field (say M), that half of the field will appear dark (fig. 18, A, 1). If the analyser be then rotated through an angle 2α so that its plane is now at right angles to the plane of the other half of the field (N), this half will now become dark (fig. 18, A, 3). When the principal plane of the analyser has the position indicated by the horizontal line through O (fig. 17), and is therefore equally inclined to the planes of polarisation of the two halves of the field, the whole field will appear uniformly faintly illuminated. This is taken as the zero position (fig. 18, A, 2).

The instrument is provided with a handle for rotating the analysing Nicol. This forms the coarse adjustment. It may be disconnected by screwing the lower ivory end of the handle downwards. When uniform, faint illumination of the field has been approximately obtained, the coarse adjustment is disconnected, and the fine adjustment put in action by screwing upwards the lower metallic end of another shorter lever. The fine adjustment lever is then moved so as to rotate the analyser by means of a small lateral screw, which is turned alternately in opposite directions until the field appears uniformly faintly illuminated to the eye of the observer. The position of the zero of the vernier in relation to

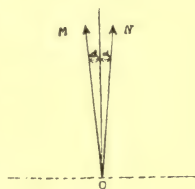


Fig. 17.

the zero of the graduated scale is then read with the aid of a pocket electric lamp and recorded. The above description refers to the adjustments found in the Landolt-Lippich three-field polarimeter. The fine adjustment must be disconnected before again using the coarse adjustment.

The analysing Nicol can be set most accurately when the angle 2α (fig. 17) is small; but the smaller the angle the less is the intensity of the light in the two halves of the field. The extent to which the angle can be reduced therefore varies with the intensity of the light and the transparency of the solution, and it is essential to have means of adjusting the value of 2α for maximal sensitiveness by means of the lever attached to the polarising Nicol P. An angle of 5° is usually fixed.

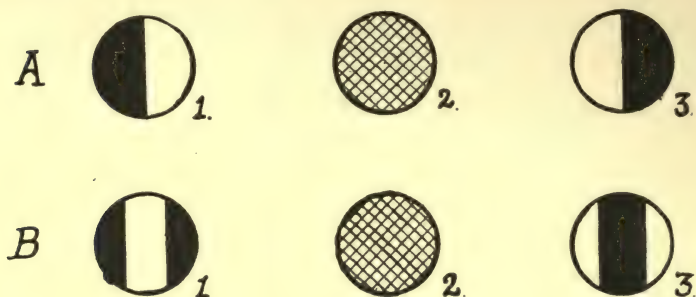


Fig. 18.

The accuracy of the Lippich polarimeter has been increased by using two lateral small Nicols in place of one. The field is therefore divided into three parts, the centre being illuminated by light that has passed through the large Nicol, and the two sides by light which has also passed through the small Nicols. The two lateral parts of the field have the same brightness. Figure 18, 1, 2, and 3 shows the appearance of the triple field for different positions of the analyser, B, 2, indicating the zero position.

Method of reading the angular rotation.

The graduated circular disc attached to the outer telescope tube rotates with the analysing Nicol past fixed verniers placed 180° apart. The position of the zero of the vernier on the scale is read by means of an adjustable lens. In the larger instruments, the graduated scale is divided into degrees, half and quarter degrees. The vernier scale is divided into 25 parts, which are equal to 24 of the 0.25° divisions of the scale, so that the vernier reads to .04 of 0.25° or to 0.01° .

Fixation of the zero position.

In rotating the polarising Nicol so as to obtain "the half shadow" angle, the zero position of the analysing Nicol is altered. If desired, the analysing Nicol can be fixed in such a position that when the zero of the vernier coincides with the zero of the scale, the field, as viewed by the eye of the observer, is uniformly faintly illuminated or set in the zero position. This adjustment is carried out by means of two small regulating screws which alter the setting of the analyser in the outer telescope tube. This adjustment is frequently omitted, as will be seen in the examples given subsequently.

I. Methods of analysing solutions containing more than one optically active substance.

The method of determining either the specific rotation of one optically active solute, when the concentration is known, or the concentration of one optically active substance, when the specific rotation is given, has already been described (pp. 20, 21, and pp. 329 to 331). When a qualitative examination of a solution has proved that two or more optically active solutes are present, their amounts can frequently be determined either by two polarimetric examinations before and after some chemical procedure, or by combining the result of a single polarimetric examination with those obtained by one or more chemical methods. The solution is first examined with the polarimeter. The result for two optically active solutes may be represented by the following "addition formula":—

$$(1) \alpha = \frac{1}{100} (c[\alpha]_D + c'[\alpha']_D)$$

α = observed rotation; l = length of observation tube in decimetres, usually 2 dm.; c = concentration of one solute; c' that of the other; and $[\alpha]_D$ and $[\alpha']_D$ the corresponding specific rotations of the two solutes: c and c' are therefore the only unknowns in the equation. If the value of c or c' or their sum can be determined by chemical means, one obtains two independent equations from which the values of c and c' may be deduced.

The "addition formula" can obviously be extended to three or more optically active solutes. If n be the number of optically active solutes of unknown concentrations, and the sum of their concentrations be also unknown, then obviously, on algebraical grounds, n independent equations will be required for their determination. The methods of obtaining these equations naturally vary according to the nature of the problem, and when the number of unknowns exceeds three the results are usually only very approximate.

The following exercises illustrate some simple applications of the formula:—

x. Method of determining whether the inversion of sucrose by boiling with dilute acids is complete or not.

Place 40 grms. of sucrose (cane sugar) in a beaker and dissolve in about 100 c.c. of hot water, cool, and quantitatively transfer the solution to a 200 c.c. volumetric flask. Wash out the beaker repeatedly with water, transfer the washings to the flask, and make up to 200 c.c.

Measure 50 c.c. of this solution with a pipette into a 100 c.c. volumetric flask of resistance glass, add 2 c.c. of N/1 HCl, mix thoroughly, and heat in the boiling water bath for 20 minutes, cool, add 1.8 to 1.9 c.c. of N/1 NaOH, and make up to 100 c.c. with water.

$[\alpha]_D^{20}$ of glucose is $+52.5^\circ$.

$[\alpha]_D^{20}$ of fructose is -92° .

$[\alpha]_D^{20}$ of sucrose is $+66.5^\circ$.

Fill a clean, dry observation tube (2 dms. long) with this solution. Fill another clean tube with water and determine the zero in the usual way. Reading for water was $+.25^\circ + .18^\circ$ (vernier reading) or $+.43^\circ$ in all (mean of three readings). Remove the tube containing water and replace it with that containing the hydrolysed sucrose. On examining this solution with the polarimeter, the observed rotation was -3.63° . The total observed *lævorotation* (α) was therefore -4.06° .

The theoretical *lævorotation* may be calculated from the formula in the following way:—1 mol or 342 grms. of sucrose yields 180 grms. of glucose and 180 grms. of fructose. The original solution contained 10 grms. of sucrose per 100 c.c. Hence, if the hydrolysis be complete, substitution of the known values in the formula gives the following result:—

$$\begin{aligned} (1). \alpha &= \frac{2}{100} \left(\frac{+52.5 \times 180 \times 10}{342} - \frac{92 \times 180 \times 10}{342} \right) \\ &= \frac{945 - 1656}{171} = -\frac{711}{171} = -4.16^\circ. \end{aligned}$$

The agreement between the observed value -4.06° and the calculated value -4.16° is sufficiently close to indicate that hydrolysis was complete; since the foregoing formula is only approximately accurate leaving out of account, as it does, the influence of concentration and temperature on the specific rotations of the sugars. The introduction of these corrections, although essential for accurate work, complicates the formula, and renders the principle of the method less evident. The corrections may therefore be omitted in class work.

If the 10 per cent. solution of sucrose be only partially hydrolysed, the foregoing formula requires to be slightly modified, thus:—

Let x be the percentage of sucrose left unaltered. Then

$$(2). \alpha = \frac{2}{100} \left(\frac{+52.5 \times 180 \times (10 - x)}{342} - \frac{92 \times 180 \times (10 - x)}{342} + 66.5x \right)$$

$$\text{Hence } x = \frac{171\alpha + 711}{298.53}.$$

EXAMPLE.

If an incompletely hydrolysed 10 per cent. solution of cane sugar be examined with the polarimeter, and α be found to be -0.67° , then the amount of sucrose, which has escaped hydrolysis, is $-.67 \times \frac{171 + 711}{298.53}$ or 2 per cent. (approximate).

Dilute acetic acid or acetic acid and sodium acetate mixtures may be used for the partial hydrolysis of cane sugar, or the hydrolysis with mineral acid may be carried out for varying periods at room temperature.

The relative amounts of the products of the action of invertase, maltase, or lactase on the sugars, sucrose, maltose, and lactose may be determined in a similar way. The polarimetric estimation of the products of enzyme action is complicated by the muta-rotation of the sugars, and by the presence of levorotatory protein. It therefore involves deproteinisation of the solutions, and abolition of muta-rotation by the addition of a trace of ammonia to the protein-free filtrates. Folin's method (see p. 376) seems well adapted for this purpose, since the acidity of the protein-free filtrates is so slight that little or no acid hydrolysis of the sugars can occur prior to the addition of ammonia. Practical work of this character is somewhat too difficult for class work, and further details are therefore omitted.

2. Estimation of sucrose in the presence of other optically active substances.

Measure 50 c.c. of 20 per cent. sucrose with a pipette into a 100 c.c. flask labelled A, add 20 c.c. of a glucose solution containing 10 grms. per 100 c.c., mix thoroughly, and make up to 100 c.c. with water.

Measure 50 c.c. of 20 per cent. sucrose solution into another 100 c.c. flask labelled B. Hydrolyse the sucrose in the way already described, cool, nearly neutralise with N/1 NaOH, add 20 c.c. of 10 per cent. glucose solution, and make up to 100 c.c. with water.

Let α be the observed rotation of solution A. Then, since the specific rotation of sucrose is $+66.5^\circ$,

$$(3). \alpha = \frac{+66.5 \times 2c.}{100} + \beta.$$

β is the rotation due to the glucose added.

An accurate formula for the specific rotation of invert sugar (Landolt) is the following:—

$$-19.7^{\circ} - .036c' + .304(t - 20).$$

Let α' be the observed rotation of solution B. Then

$$(4). \alpha' = \frac{2c'}{100} \left[-19.7^{\circ} - .036c' + .304(t - 20) \right] + \beta$$

c' is the concentration of invert sugar, or grammes per 100 c.c., and t is the temperature.

Since 1 mol or 342 grms. of sucrose ($C_{12}H_{22}O_{11}$) yields 1 mol or 180 grms. of glucose and 1 mol or 180 grms. of fructose, the concentration c' of invert sugar is $\frac{360c}{342}$ or 1.053c.

The subtraction of (4) from (3) gives,

$$(5). \alpha - \alpha' = \frac{66.5 \times 2c}{100} - \left[-19.7 - .036 \times 1.053c + .304(t - 20) \right] \frac{2c \times 1.053}{100}$$

All the terms in this expression are known with the exception of c , which can therefore be calculated. Since β is eliminated, it is obvious that this method can be used in determining the amount of sucrose in a solution containing other optically active substances, if the latter are not affected by boiling with dilute acid. When given as a class exercise, the quantity of cane sugar is assumed to be unknown.

3. Combined polarimetric and chemical methods.

EXAMPLE.

Determination of percentages of glucose and fructose in a solution containing the sugars in unknown proportions. Such solutions may be prepared by adding a known quantity of glucose to a solution of invert sugar.

Place the solution in a 2 dm. observation tube and examine with the polarimeter. On applying formula (1) an equation is obtained containing c and c' (the concentrations of the two sugars) as unknowns.

With a pipette measure 10 c.c. of the solution into a 100 c.c. flask, make up to 100 c.c. with water, and determine the percentage of reducing sugar expressed as glucose by Benedict's method (pp. 327 and 328). If less than 10 c.c. of this dilute solution are required, dilute to a suitable strength and repeat the titration.

After allowing for extent of dilution, one obtains an equation of the form

$$c + 0.962c' = p;$$

c and c' are concentrations or grammes of glucose and fructose per 100 c.c. of the solution. The concentration of fructose c' is multiplied by 0.962 because .052 grms. fructose has the same reducing power as .05 grms. glucose, and p is stated as percentage of glucose.

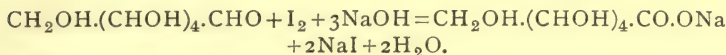
The polarimetric observations give another independent equation containing c and c' . By solving these equations the values of c and c' are obtained.

4. Solutions containing other sugars may be analysed in a similar way by combining the results of polarimetric and reduction or iodometric methods.¹

II. Willstätter and Schudel's iodometric method of estimating sugars.²

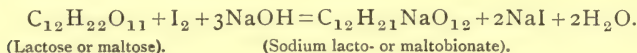
This recent method, which is a modification of one originally proposed by Romijn, seems likely to prove of great value in analysing solutions containing several sugars, especially when combined with the polarimetric method.

The method is based on the following considerations:—Aldoses are oxidised by sodium hypoiodite under the conditions determined by W. and S. in the way represented by the equation (see p. 12).



Neither ketoses nor sucrose are appreciably oxidised under these conditions.

The disaccharides, maltose and lactose, appear to be oxidised in the way represented by the following equation:—



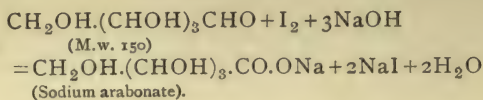
According to these equations, 1 c.c. of N/10 iodine is equivalent to 1 c.c. of a M/20 solution of an aldose. 1 c.c. of N/10 iodine is therefore theoretically equivalent to 0.009 gm. of anhydrous glucose, galactose, or mannose (? Judd finds that mannose is oxidised with greater difficulty than the other sugars), or to 0.0099 gm. of these sugars when containing one molecule of water of crystallisation. Similarly, 1 c.c. of N/10 iodine is equivalent to 0.018 gm. of lactose or maltose ($\text{C}_{12}\text{H}_{22}\text{O}_{11} \cdot \text{H}_2\text{O}$), or to 0.0171 gm. of the anhydrous sugars. After inversion, sucrose reduces the hypoiodite to an extent proportional to the amount of glucose formed. After complete hydrolysis 0.0171 gm. of sucrose is therefore equivalent to 1 c.c. of N/10 iodine.

1 c.c. of N/10 iodine is theoretically equivalent to 0.0075 gm. of an

¹ For details the reader is referred to works on sugar analysis, such as "A Handbook of Sugar Analysis" (pp. 472 to 493), by C. A. Browne. New York: J. Wiley & Sons.

² Literature:—(1) "Ber. d. Deutsch. Chem. Ges.," 51, pp. 780-781; (2) J. Bang, "Bioch. Zeitschr.," 92, 344-346; (3) H. M. Judd, "Bioch. Journal," xiv. 255-262; (4) Baker & Hulton, "Bioch. Journal," xiv. 754 to 756.

aldo-pentose ($C_5H_{10}O_5$). For example, arabinose is probably oxidised thus :—



Willstätter and Schudel have found that the most accurate results are obtained under the following conditions:—Add to a portion of the solution of sugar (say 10 c.c.) about twice the amount of N/10 iodine required by the equation, and about $1\frac{1}{2}$ times as much N/10 NaOH as iodine. Allow the solution to stand for 12 to 15 minutes (3 to 5 minutes are sufficient according to Baker and Hulton). Then acidify with an amount of N/1 or N/10 H_2SO_4 equivalent to the quantity of N/10 caustic soda originally added, and titrate the excess of iodine with N/10 or N/20 sodium thiosulphate, using starch solution as indicator.

Let y be the number of c.c. of N/10 thiosulphate required and x the number of c.c. of N/10 iodine originally added, then, if the sugar be an anhydrous aldohexose, the percentage of sugar is $0.09(x - y)$.

When one is dealing with a sugar solution of unknown concentration, one or, if necessary, two approximate estimations are carried out, using 1 c.c. of the sugar solution. One can ascertain from this approximate result whether the sugar solution requires to be diluted or not, and also how much N/10 iodine will be required to fulfil the conditions prescribed by W. and S.

EXAMPLES.

(A) Add 20 c.c. of N/10 iodine to 10 c.c. of a solution of glucose containing 1.00 gm. of the anhydrous sugar per 100 c.c. of solution. Then add 30 c.c. of N/10 NaOH, and allow the solution to stand for 10 minutes. Add 3 c.c. N/1 H_2SO_4 , titrate the free iodine with N/10 sodium thiosulphate until the colour is nearly discharged, add a few drops of a 1 per cent. solution of soluble starch, and add the thiosulphate solution drop by drop until the blue colour is just discharged; 9 c.c. were required. Hence $(20 - 9) \times .09$ or 0.99 per cent. of glucose was found.

(B) ESTIMATION OF SUCROSE.

2 c.c. of N/10 HCl were added to 10 c.c. of a solution of sucrose containing 1.7373 grms. per 100 c.c., and the solution was heated for 15 minutes in a flask immersed in boiling water. The solution was cooled, mixed with 20 c.c. N/10 iodine, and 32 c.c. N/10 NaOH, and allowed to stand 15 minutes. 30 c.c. of N/10 H_2SO_4 were added, and the free iodine titrated with N/10 sodium thiosulphate; 9.7 c.c. of the latter were required. Hence the percentage of cane sugar was found to be 10.3×0.171 or 1.761.

The results for some other sugars are given in the following table :—

N/10 Iodine absorbed by 10 c.c. of the solution.	Per cent. found.	Theoretical per cent.	Sugar used.
9.9 c.c.	0.980	0.9675	Galactose ($C_6H_{12}O_6 \cdot H_2O$)
5.4	0.972	0.961	Lactose ($C_{12}H_{22}O_{11} \cdot H_2O$)
9.4	1.692	1.677	Maltose (")
N/100 Iodine absorbed by 10 c.c.			
10.45 c.c.	0.0784	0.0773	Arabinose ($C_5H_{10}O_5$).
12.3	0.1107	0.1102	Glucose ($C_6H_{12}O_6$).

An iodometric estimation of mannose in aqueous solution using N/100 iodine and N/100 thiosulphate gave results greatly divergent from the theoretical. The theoretical percentage of mannose was 0.0881 gm., the percentages found were 0.0657 (after 20 minutes standing), and 0.0693 (after 1 hour 20 minutes standing with hypiodite).

The iodometric method cannot be applied to the estimation of sugars in solutions, such as diabetic urine, containing many other organic substances, which reduce hypiodites.

J. Bang has applied the method to protein-free filtrates from blood, and obtained values approximately double those found for glucose by his method of micro-analysis. The substances which form the source of the non-protein nitrogen of the blood and lipins are probably mainly responsible for this result. In pathological cases, acetone and allied bodies would also absorb relatively large quantities of iodine. These facts do not exclude the possibility that the iodometric method, if applied to protein-free blood filtrates in health and disease, might yield valuable results. If a value, which might be termed "the iodine index," giving the number of c.c. of N/100 iodine absorbed by the protein-free filtrate from 100 cc. or grms. of blood were determined in normal and in pathological cases, the results obtained might throw light on the condition of the blood in cases of diabetes, and such pathological states as uræmia. The simplicity of the technique of the iodometric method renders it suitable to form the basis of a clinical micro-analytical method.

III. The following important applications of the polarimeter in biological chemistry can only be briefly referred to :—

(1) **Determination of the velocity constants of the hydrolysis of disaccharides by acids and by enzymes.**

The reader is referred to text-books of physical chemistry for descriptions of technique.¹

¹ See also S. Arrhenius, "Quantitative Laws in Biological Chemistry," chapter ii. London: Bell & Sons.

(2) Biochemical identification of glucosides with the aid of emulsin (E. Bourquelot).

Principle of Method.—All the known glucosides, which are hydrolysed by emulsin, are laevorotatory derivatives of glucose. Hence, on hydrolysis the solutions become dextro-rotatory owing to the setting free of glucose. In the case of salicin ($[\alpha]_D = -64.9^\circ$), the one product of hydrolysis, saligenin, is optically inactive, while the other product, glucose, is dextro-rotatory. The extent and rate of hydrolysis can, therefore, be determined by polarimetric observations.

After hydrolysis, the amount of glucose is determined by titration. The ratio of the observed alteration in rotation to the amount of glucose has been found to be constant for any given β -glucoside. The value of the ratio is characteristic for each β -glucoside, and can therefore be utilised for the identification of glucosides in plants.

(3) Demonstration of the synthetic action of enzymes.

The chief difficulty in the practical study of the synthetic action of enzymes depends on the fact that the action is a slow one, and polarimetric and chemical observations have therefore to extend over a considerable period varying from 10 to 80 days. This circumstance renders such experiments unsuitable for class work.

The reader is referred to the follow-

ing books for details and references to the literature of the subject:—E. F. Armstrong, "The Simple Carbohydrates and Glucosides;" W. M. Bayliss, "The Nature of Enzyme Action," London: Longmans.

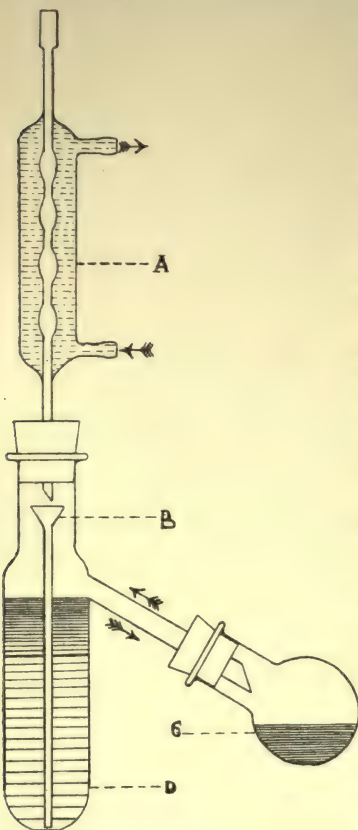


Fig. 19.

IV. Polarimetric estimation of β -hydroxybutyric acid in pathological urine (Embden and Schmitz's method).

Measure with a pipette 100 c.c. of urine into the vessel D (fig. 19), add 80 to 90 grms. of finely powdered ammonium sulphate and 5 c.c. of

25 per cent. sulphuric acid. Shake from time to time until the salt has almost completely dissolved. Then fit up the apparatus in the manner indicated in the figure, placing purified ether in the flask. Place the flask on a water bath heated by a small flame, the burner being completely surrounded with wire gauze. The ether vapour is condensed in A, drops into B, and then passes upwards through the fluid in D. The lower opening of B should have a bulbous end perforated with small openings. In some modifications of this apparatus, means are provided for mechanical stirring of the fluid in D. When the condensed ether reaches the level of the wide side tube, it overflows into the flask. If the quantity of ether placed initially in the flask be insufficient, more may be gradually added through the central tube of the condenser. Allow the extraction to continue for 24 hours, then quantitatively transfer the ethereal extract to a distillation flask, add 5 c.c. of water, and distil off the ether at as low a temperature as possible. Cool the aqueous residue, transfer quantitatively to an accurate, small measuring cylinder, and make up to 10 to 15 c.c. with the washings from the distillation flask. Add a small quantity of pure animal charcoal, shake thoroughly, filter, and examine the filtrate with the polarimeter, using a one or two decimeter tube.

Let α be the observed rotation. The specific rotation of β -hydroxybutyric acid $[\alpha]_D^{20}$ is -24.12° . Hence $c = \frac{100\alpha}{24.12}$; c is the number of grammes of β -hydroxybutyric present in 100 c.c. of the aqueous solution. The total quantity of solution being known, the amount of β -hydroxybutyric present in it may, therefore, easily be calculated. The value obtained is the amount of β -hydroxybutyric acid present in the 100 c.c. of urine taken.

The apparatus used for this extraction is also employed in Dakin's method for the separation of the amino-acids (p. 137).

The reader is referred to larger practical text-books for further applications of the Polarimeter, e.g. "Das Arbeiten mit optisch-aktiv. Kohlenstoffverb," by E. Eichwald ("Abderhalden's Handbuch," vol. ix. pp. 625 to 664).

V. Micropolarisation (same Handbuch, vol. v. p. 572). E. Fischer worked out the technique of a micro-analytical method, which renders possible the polarimetric examination of small quantities of fluid, such as 0.1 c.c., and which is therefore of great value in Biological Chemistry.

CHAPTER XXIV

COLORIMETRIC METHODS IN PHYSIOLOGICAL CHEMISTRY

If two solutions containing the same colouring matter in different concentrations be examined in layers, the thicknesses of which are inversely proportional to the concentrations of the solute, then the two layers will be found to absorb the same amount of light, and to have, therefore, the same colour and brightness.

A method of optical analysis of colouring matters has been based on this principle. The thickness of layer of the standard solution being fixed, and the concentration of the solution known, the thickness of the solution of unknown concentration is altered until the colours of the two solutions appear to be the same.

If c represent the concentration of the standard solution, c' that of the solution of unknown concentration, and h and h' the corresponding thicknesses of the two solutions, then $\frac{c'}{c} = \frac{h}{h'}$ or $c' = \frac{ch}{h'}$, c and h being known, and h' having been measured, c' can be readily calculated from the formula.

The instruments, by means of which such colour comparisons are carried out, are known as colorimeters. The colorimeter which has been most frequently used in physiological chemistry is that of Duboscq. It consists of two outer glass cylinders closed below by plane glass plates (fig. 20). The standard solution is placed in one of these, and the solution of unknown concentration in the other. The thicknesses of the layers are varied by raising or lowering two inner glass tubes or plungers (T and T_1), closed at their lower ends by plane glass plates. The movement of each plunger is controlled by a screw, the rotation of which gives an independent vertical motion to each plunger. The distances from the bottoms of the plungers to the bottoms of the outer cylinders can be measured to 0.1 mm. by means of millimeter scales and verniers.

Since the eye is somewhat insensitive to differences of light intensity and quality, it is essential that the two coloured fields should be brought into optical contact, the line of demarcation being reduced to a minimum. This is secured in the case of the Duboscq instrument by the optical

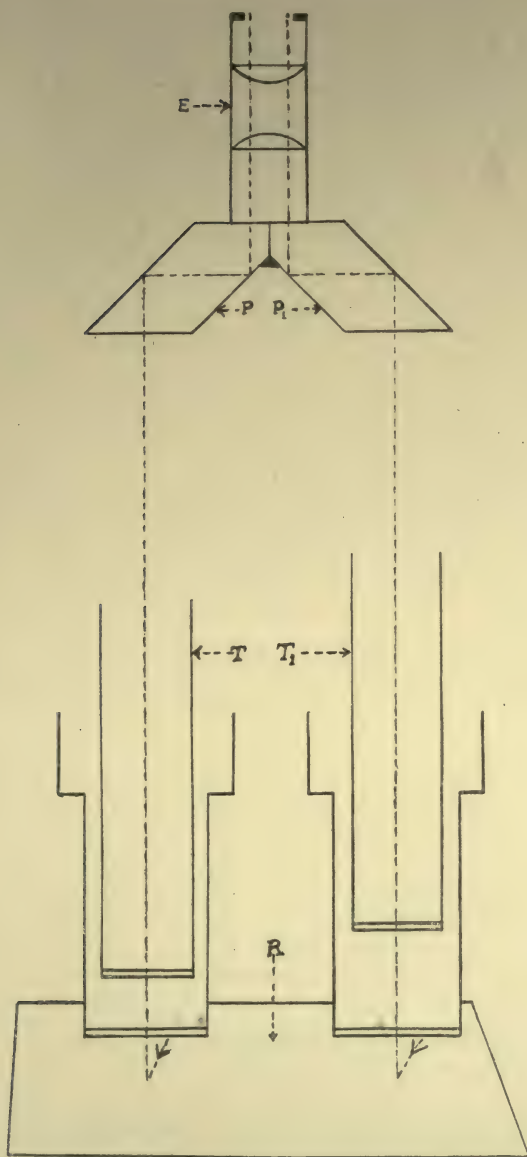


Fig. 20.

arrangements shown in figure 20. The rays of light which have passed through the two tubes are twice reflected in the prisms P and P₁, and brought into optical contact. On looking through the eyepiece, the observer sees a circular field, one-half of which is illuminated by light which has passed through one solution and the plunger T, and the other by light which has passed through the other solution and the plunger T₁. When the two halves of the field have been matched as closely as possible by altering the thickness of layer of the solution of unknown concentration, the line of separation between the two halves of the field should nearly vanish. This result appears to be more fully attained with colorimeters based on the principle of Lummer-Brodhun photometers than with the Duboscq instrument. A description of such colorimeters is omitted since they have not yet been extensively used in physiological chemistry, and the writer's experience has been confined to the Duboscq instrument. Still more accurate results might be obtainable with colorimeters based on the principle of the Lummer-Brodhun contrast photometer.¹

Adjustment of the colorimeter.

See that the lenses, prisms, and tubes are clean. Control the accuracy of the scales by carefully lowering the plungers until their lower ends just come into contact with the bottoms of the outer vessels. If the zeroes of the verniers do not coincide with the zeroes of the scales, all the subsequent observations must be corrected accordingly.

The colorimeter may be adjusted with water in the outer tubes. According to Folin, the instrument should be placed in the middle of the laboratory, so that the eye is not dazzled by direct light from the window.

Focus the line of separation between the two halves of the field with the eyepiece. Secure equal illumination of the two halves by moving the instrument as a whole or rotating the reflecting surface R.

The two solutions, which are to be compared, should be of nearly the same concentration in order to minimise the effect of absorption of light by the solvent, and also to prevent any differences in absorption due to association or dissociation of the molecules of the colouring matter, which might occur, if the concentrations of the two solutions were markedly different.

Both solutions should be transparent, and should have the same temperature.

Some quantitative colorimetric methods.

I. Creatinine in urine (Folin).

REAGENTS AND APPARATUS REQUIRED.

(1) *Standard solution of creatinine. Dissolve 1.6106 grms. of the pure creatinine zinc chloride compound in water, transfer quanti-*

¹ For fuller details regarding the optical construction of colorimeters, the reader is referred to:—(1) Donnan, "Zeitschr. Physik. Chem.," xix. p. 465. (2) Kober, "J. of Biol. Chem.," xxix. p. 155. (3) G. and H. Krüss, "Kolorimetrie," Hamburg: L. Voss.

tatively to a litre flask, add 100 c.c. of $N/1$ HCl , and make up to a litre with water. 1 c.c. contains 1 mgrm. of creatinine.

(2) 10 per cent. caustic soda (to be measured from a burette).

(3) A saturated aqueous solution of pure picric acid (about 1 per cent.).

(4) Ostwald pipettes of 1 or 2 c.c.

(5) 20 c.c. pipette.

(6) Two 100 c.c. stoppered measuring flasks.

Measure with an Ostwald pipette 1 or 2 c.c. of urine into a 100 c.c. flask, and 1 c.c. of the standard creatinine into another 100 c.c. flask. Measure 20 c.c. of solution (3) into each flask, then add from a burette 1.5 c.c. of (2), and let stand for 10 minutes. Then make up each solution to 100 c.c., and mix thoroughly.

"Read the standard against itself in the colorimeter at 20 mm. until the correct value can be obtained. The error in reading should not exceed 0.2 mm. Rinse the right hand cup with the unknown, and determine its colour in terms of the standard set at 20. Let the reading be h' mm. Then, $20/h'$ gives the creatinine in mgrms. in the quantity of urine taken."

Ia. Creatinine in urine.—(Folin's original method with $N/2$ potassium bichromate as standard solution). This method is less accurate than I.; but, when pure creatinine zinc chloride is not available, may replace the method just described.

REAGENTS AND APPARATUS REQUIRED.

(1) $N/2$ potassium bichromate solution. Place 24.55 grms. of potassium chromate in a litre flask, dissolve in water, and make up the solution to a litre.

(2) 10 c.c. pipette; and (3) two burettes.

PROCEDURE.

Place 10 c.c. of urine in a 500 c.c. measuring flask, add 15 c.c. of saturated picric acid solution, and 5 c.c. of 10 per cent. sodium hydrate solution. Mix thoroughly and allow to stand for 5 minutes.

Place some $N/2$ bichromate solution in the two outer cylinders of the colorimeter, set the depth of one to 8 mm., and adjust the depth of the other until the two halves of the coloured field are accurately matched. The error in reading should not exceed 0.2 mm. from the true value, 8 mm. Fill up the solution in the 500 c.c. flask with water to the mark. Wash out the right hand cup repeatedly with water, then rinse out two or three times with the solution from the 500 c.c. flask, then place some of this solution in the cup, and determine its colour in terms of the standard set at 8 mm.

CALCULATION.

Folin found that 10 mgrms. of pure creatinine, when dissolved in water in a 500 c.c. flask and treated in the way described for urine, gives a solution, 8.1 mm. thickness of which possesses the same colorimetric value as 8 mm. of $N/2$ solution of potassium bichromate. Let h' mm. be the reading for the 10 c.c. sample of urine treated as

described, then the quantity of creatinine in mgrms. in 10 c.c. of the urine taken is $\frac{10 \times 8.1}{h'}$. If the value thus obtained be above 15 mgrms., or below 5 mgrms., the estimation should be repeated with a proportionally smaller or larger volume of urine than 10 c.c.

II. Creatine (Folin).

The urine of normal adults contains only traces of creatine. Relatively considerable quantities are present in the urine of children, and in that of adults in certain pathological conditions. Creatine is converted into creatinine and estimated as the latter base.

PROCEDURE.

"Measure the urine (usually 1 c.c.) into a flask (capacity 300 c.c.), and add 20 c.c. saturated picric acid solution. Weigh flask and contents and add about 150 c.c. water. Boil gently for 45 minutes, then more rapidly until the original volume within 3 or 4 grms. (determined by weighing) is obtained. Cool, add 1.5 c.c. 10 per cent. sodic hydrate, let stand 10 minutes, and compare, as in the case of preformed creatinine, with the colour obtained from 1 mgrm. creatinine.

"Twenty divided by the reading gives the sum of the creatine and creatinine present.

"Calculate the total quantity and subtract the preformed creatinine."

The autoclave method of converting creatine into creatinine is more rapid and convenient.

"Measure 1 c.c. urine into a 100 c.c. volumetric flask, and 20 c.c. saturated picric acid solution. Cover the mouth of the flask with tin-foil, and heat in the autoclave at 115°-120° for 20 minutes. Cool, add 1.5 c.c. sodic hydrate, and finish the determination in the usual manner."

III. Glucose in Blood (Folin and Wu).

i. Preparation of protein-free blood filtrates.

REAGENTS AND APPARATUS REQUIRED.

(1) 10 per cent. solution of pure sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) not containing an excess of sodium carbonate. The carbonate content may be determined as follows:—Add one drop of phenolphthalein (1 per cent.) to 10 c.c. of the sodium tungstate solution, and titrate with N/10 HCl. The amount of acid required should not exceed 0.4 c.c.

(2) Two-thirds normal sulphuric acid. Dilute 35 grms. of concentrated chemically pure sulphuric acid with water up to a litre. Check the concentration of the acid by titration with N/1 NaOH. "The acid solution is intended to be equivalent to the sodium content of the tungstate so that, when equal volumes are mixed substantially,

the whole of the tungstic acid is set free without the presence of an excess of sulphuric acid. The tungstic acid set free is nearly quantitatively taken up by the proteins, and the blood filtrates are therefore only slightly acid to congo red." If excess of sulphuric acid be added, part of the uric acid present in the blood is precipitated along with the proteins.

(3) Three burettes containing water, 10 per cent. sodium tungstate, and 2N/3 sulphuric acid.

(4) 100 c.c. flask with rubber stopper.

(5) Special graduated blood pipette of 15 c.c. capacity.

PROCEDURE.

"The blood is collected over finely powdered potassium oxalate (about .02 gm. per 10 c.c. blood). Transfer 5 c.c. (5 to 15 c.c. may be taken) of oxalated blood to a 100 c.c. flask (capacity of flask 15 to 20 times the volume of blood taken). Liquefy the blood with 7 volumes (35 c.c.) water. Add one volume (5 c.c.) of 10 per cent. sodium tungstate, and mix. Add slowly, and with shaking, one volume (5 c.c.) of 2N/3 H_2SO_4 . Close the mouth of the flask with a rubber stopper, and shake. Let stand for 5 minutes. The colour of the coagulum gradually changes from bright red to brown. If this change in colour does not occur owing to coagulation being incomplete (usually the result of excess of oxalate), add 10 per cent. sulphuric acid, one drop at a time, shaking vigorously after each drop, and continuing until there is practically no foaming and the coagulum is brown."

Pour the mixture on a filter paper large enough to hold it all, beginning the filtration by adding a few c.c. down the double portion of the filter paper. When the whole filter paper is wet, pour the whole of the mixture into it, and cover the funnel with a watch-glass. The filtrate should be as clear as water, and should not require refiltering.

The blood filtrate is nearly neutral. 10 c.c. of it require about 0.2 c.c. N/10 NaOH for neutralisation (using phenolphthalein as indicator).

If the filtrate requires to be kept for some time, add a few drops of toluene or xylene to prevent bacterial decomposition.

The protein free filtrate may be used for the determination of non-protein nitrogen, urea, uric acid, creatinine, creatine, and sugar.

2. Determination of sugar in a protein-free blood filtrate (Folin and Wu).

REAGENTS REQUIRED.

(1) Saturated sodium carbonate solution.

(2) Alkaline copper solution.

"Dissolve 40 grms. of anhydrous sodium carbonate in about 400 c.c. of water, and transfer to a litre flask. Add 7.5 grms. of tartaric acid, and, when the latter has dissolved, add 4.5 grms. of crystallised copper sulphate, mix, and make up to a litre."

(3) *Phosphotungstic-phosphomolybdic acid.* Place 25 grms. of molybdenum trioxide (MoO_3) or 34 grms. of ammonium molybdate $[(\text{NH}_4)_2\text{MoO}_4]$ in a large flask (1.5 to 2 litres), add 140 c.c. of 10 per cent. sodium hydrate solution, and about 150 c.c. water. Boil for at least 20 minutes to drive off the ammonia, until the steam from the flask no longer reacts alkaline to red litmus paper. Add to the solution 100 grms. of sodium tungstate, 50 c.c. of 85 per cent. phosphoric acid, and 100 c.c. of concentrated hydrochloric acid. Dilute to a volume of 700 to 800 c.c., and close the mouth of the flask with a funnel and watchglass. Boil gently for not less than 4 hours, adding hot water from time to time to replace that lost by evaporation. Cool, transfer quantitatively to a litre flask, and dilute to one litre. "This solution is identical with the phenol reagent of Folin and Denis. For use in the determination of blood sugar, dilute 1 volume (100 c.c.) of the reagent with one-half volume (50 c.c.) of water, and one-half volume (50 c.c.) of concentrated hydrochloric acid."

(4) *Standard sugar solution.* Dissolve exactly 1 gm. of pure anhydrous glucose in water and make up to 100 c.c. in a volumetric flask. Transfer to a bottle, add a few drops of toluene or xylene, shake, and stopper tightly.

If pure glucose is not available, place exactly 1 gm. of cane sugar in a 100 c.c. measuring flask, add 20 c.c. of $\text{N}/1$ HCl , and let the solution stand overnight at room temperature (or rotate the flask and contents continuously for 10 minutes in a water bath kept at 70°). Add 1.68 grms. sodium bicarbonate, and about 0.2 gm. sodium acetate, shake for a few minutes to get rid of most of the carbon dioxide, and make up to 100 c.c. with water. Then add 5 c.c. of water (1 gm. cane sugar yields 1.05 grms. of invert sugar) and mix. Store this stock solution in the same way as that of glucose, adding toluene or xylene. Both solutions keep indefinitely.

These stock solutions are diluted for the determination of sugar in the following way:—Measure 5 c.c. of the stock solution into a 500 c.c. volumetric flask, make up to 500 c.c., and mix thoroughly. 10 c.c. of the diluted solution contain 1 mgrm. of glucose or invert sugar.

APPARATUS REQUIRED.

- (1) Beaker or mug containing boiling water to serve as water bath.
- (2) Three pipettes to deliver 2 c.c., and one graduated pipette.
- (3) Two test-tubes (200×20 mm.) graduated at 25 c.c.

METHOD OF ANALYSIS.

Measure 2 c.c. of the blood filtrate into one test-tube, and 2 c.c. of the dilute standard sugar solution into the other. Add to the contents of each tube 2 c.c. of the alkaline copper solution, and immerse the tubes in boiling water for 6 minutes. Remove the test-tubes and add to each at once without cooling 1 c.c. (measured with a graduated pipette, e.g. 5 c.c. pipette graduated to .05 c.c.) of the acidified and diluted phenol reagent. The hydrochloric acid is added to dissolve the cuprous oxide. Mix, cool, and add 5 c.c. of the saturated sodium carbonate solution. An intense blue colour

gradually develops, which will remain unaltered for several days Dilute the contents of both test-tubes to the 25 c.c. mark, and after at least 5 minutes make the colour comparison.

"The depth of the standard (in mm.) multiplied by 100 and divided by the reading of the unknown gives the sugar content, in mgrms., per 100 c.c. of blood."

SLIGHTLY MODIFIED PROCEDURE.

If sugar is the only substance to be estimated in the blood filtrate, a smaller quantity of blood may be used.

Place 5 c.c. of water in an accurately graduated 25 c.c. cylinder provided with a glass stopper. Draw up 1 c.c. of oxalate blood into a 1 c.c. pipette (measured to contain). Cautiously remove adherent blood from the surface and point of the pipette with filter paper, and immerse the lower end of the pipette in the water in the cylinder so that the opening of the pipette lies fully halfway below the surface. Expel the blood by blowing gently into the pipette, and wash out the pipette by sucking water into it and again expelling the water at least twice. Finally withdraw the pipette, blowing out the last traces of fluid while the point of the pipette is in contact with the inner surface of the cylinder. Make up the solution in the cylinder to 8 c.c., add 1 c.c. of 10 per cent. sodium tungstate solution, and add drop by drop from a burette 1 c.c. of $2N/3$ H_2SO_4 , shaking after each addition. Mix thoroughly and follow the further procedure already described.¹

3. Determination of creatine plus creatinine in blood (Folin and Wu).

A. REAGENTS REQUIRED.

(1) $N/1$ HCl . (2) Saturated aqueous solution of pure picric acid (about 1 per cent.). (3) 10 per cent. sodium hydrate. (4) Alkaline picrate solution (freshly prepared). Measure 25 c.c. of (2) into a small flask, add 5 c.c. of (3), and mix. (5) Standard creatinine solution (for blood). Measure 6 c.c. (= 6 mgrms. creatinine) of the standard solution used for urine analysis into a litre flask, add 10 c.c. $N/1$ HCl , make up to a litre with water, and mix. Transfer to a bottle and add 4 or 5 drops of toluene as a preservative. 5 c.c. of this solution contain 0.03 mgrm. creatinine.

B. APPARATUS REQUIRED.

- (1) One 1 c.c., two 5 c.c., and two 10 c.c. pipettes.
- (2) Two test-tubes graduated at 25 c.c.
- (3) One 50 c.c. volumetric flask.
- (4) Autoclave.

¹ Literature:—(1) Folin and Wu, "J. Biol. Chem.," xxxviii. p. 106, and xli. p. 367. (2) Mackenzie Wallis, and Gallagher (1920), "Lancet" (October), p. 784. (3) E. Ponder and L. Howie, "Biochem. J.," xv. p. 171. Papers (2) and (3) contain microchemical modifications of Folin and Wu's method.

C. METHOD OF ANALYSIS.

Measure 5 c.c. of the protein-free blood filtrate into a test-tube graduated at 25 c.c., add 1 c.c. of $N/1HCl$, cover the mouth of the test-tube with lead foil, and heat in the autoclave to 130° for 20 minutes or to 155° for 10 minutes. In the meantime, prepare the standard creatinine solution by measuring 10 c.c. of the creatinine solution (5) into a 50 c.c. flask, adding 2 c.c. of (1) and 10 c.c. of solution (4), and, after 10 minutes standing, diluting to 50 c.c. with water.

Remove the other test-tube from the autoclave, cool completely, add 5 c.c. of solution (4), let stand for 10 minutes, then dilute to 25 c.c. with water, and mix. Make the colorimetric comparison in the usual way.

CALCULATION.

Multiply the height of the standard (usually 20 mm.) by 6 and divide by the height in mm. of the unknown. The result gives the total creatinine in mgrms. per 100 c.c. blood.¹

A large number of other colorimetric methods have been devised. For fuller details the reader is referred to such books as (1) O. Folin, "Laboratory Manual of Biological Chemistry," New York and London: D. Appleton; and (2) P. B. Hawk, "Practical Physiological Chemistry."

¹ "The normal value for total creatinine given by this method is about 6 mg. per 100 c.c. of blood.

"In the case of uræmic bloods containing large amounts of creatinine, 1, 2, or 3 c.c. of blood filtrate, plus water enough to make approximately 5 c.c., are substitutes for 5 c.c. of the undiluted filtrate."

SECTION III
APPENDIX



I

TABLE OF REAGENTS¹



1. ACIDS

(A) MINERAL

HYDROCHLORIC ACID, CONCENTRATED (Sp. Gr. 1.16 to 1.19).

HYDROCHLORIC ACID, .25 per cent.—8 c.c. of the concentrated acid to 992 c.c. of water.

NITRIC ACID, CONCENTRATED PURE (Sp. Gr. 1.42).

NITRIC ACID, FUMING (Sp. Gr. 1.5).

SULPHURIC ACID, CONCENTRATED (Sp. Gr. about 1.841).

(B) ORGANIC

ACETIC ACID, GLACIAL (Sp. Gr. 1.06. 100 c.c. contain 111.1 grms.).

ACETIC ACID, 33 per cent.

ACETIC ACID, 2 per cent.

TRICHLORACETIC ACID, 5 per cent.

OXALIC ACID, SATURATED AQUEOUS SOLUTION.

PICRIC ACID, SATURATED AQUEOUS SOLUTION.

TARTARIC ACID, SATURATED AQUEOUS SOLUTION.

GLYOXYLIC ACID (see SPECIAL TEST SOLUTIONS).

2. ALKALIES

SODIUM HYDRATE, CONCENTRATED (Sp. Gr. 1.34).

SODIUM HYDRATE, 10 per cent. (by volume).

POTASSIUM HYDRATE, 10 per cent. (by volume).

AMMONIA, CONCENTRATED (Sp. Gr. .88).

3. SALTS

AMMONIUM MOLYBDATE.—*Dissolve 50 grms. of molybdic acid in 200 grms. of 10 per cent. ammonia. Gradually pour this solution into 750 grms. of nitric acid (sp. gr. 1.2), shaking vigorously after each addition. Allow the solution to stand for a few days, and pour it off from the sediment which forms.*

¹ The composition and methods of preparation of a number of reagents have been given previously in the text of this book.

I—CONTINUED

AMMONIUM OXALATE, 5 per cent.

AMMONIUM SULPHATE, SATURATED SOLUTION.

AMMONIUM SULPHATE, SOLID.

AMMONIUM SULPHIDE SOLUTION.

BARIUM CHLORIDE, 10 per cent.

BARYTA MIXTURE.—*Two volumes of a saturated aqueous solution of barium hydrate and one volume of a saturated solution of barium nitrate or chloride.*

CALCIUM CHLORIDE, 2.8 per cent.

COPPER SULPHATE, 1 per cent.

FERRIC CHLORIDE, 2 per cent.

LEAD ACETATE, 10 per cent.

BASIC LEAD ACETATE.—*Boil lead acetate solution with excess of lead oxide, and filter.*

MAGNESIUM SULPHATE, SOLID.

MAGNESIUM SULPHATE, SATURATED SOLUTION.

MAGNESIA MIXTURE.—*Magnesium chloride (pure) 110 grms., ammonium chloride 140 grms., ammonia (sp. gr. .91) 250 c.c., and water 1750 c.c.*

MERCURIC CHLORIDE, SATURATED SOLUTION.

POTASSIUM FERRICYANIDE, SATURATED SOLUTION.

POTASSIUM FERROCYANIDE, 5 per cent.

POTASSIUM SULPHATE, ANHYDROUS.

POTASSIUM SULPHOCYANATE, 5 per cent.

SILVER NITRATE, 2 per cent.

SODIUM ACETATE, SOLID.

SODIUM ACETATE, ACID SOLUTION.

(Sodium acetate, 10 grms.; glacial acetic acid, 3 grms.; distilled water to 100 c.c.)

SODIUM CARBONATE, 10 per cent.

SODIUM CARBONATE, 2 per cent.

SODIUM CHLORIDE, SOLID.

SODIUM CHLORIDE, SATURATED SOLUTION.

SODIUM HYPOBROMITE (FOR HUFNER'S METHOD).

Dissolve 100 grms. NaOH in 250 c.c. distilled water, and add 25 c.c. of bromine.

SODIUM SULPHATE, ANHYDROUS SOLID.

4. ORGANIC SOLVENTS

METHYLATED SPIRIT.

ABSOLUTE ETHYL ALCOHOL (Sp. Gr. at 20° is 0.789; B.p. at 760 mm. Hg. 78.3°).

METHYL ALCOHOL (Sp. Gr. at 20°, 0.796; B.p. at 760 mm., 67°).

AMYL ALCOHOL (Sp. Gr. at 20°, 0.812; B.p. at 760 mm., 129 to 132).

BUTYL ALCOHOL (B.p. of isobutyl, 108.4°; Sp. Gr. at 20°, about 0.81).

I—CONTINUED

ACETONE (Sp. Gr. at 20° is 0.7920; B.p. at 760 mm., 56.5°).
ACETIC ACID, GLACIAL (Sp. Gr. at 20° is 1.0497; B.p., 118°; M.p., 16.7°).
ACETIC ANHYDRIDE (Sp. Gr. at 20°, 1.073; B.p., 137°).
BUTYRIC ACID, NORMAL (Sp. Gr. at 20°, 0.96; B.p. 163°).
ETHYL ETHER (Sp. Gr. at 0° 0.736; B.p., 35°; solubility in water, 1 in 10).
CHLOROFORM (Sp. Gr. at 15°, 1.5; B.p., 61.5°).
CARBON TETRACHLORIDE (Sp. Gr. at 0°, 1.631; B.p., 78.5°).
CARBON BISULPHIDE (Sp. Gr. at 0°, 1.297; B.p., 47°).
TOLUENE (Sp. Gr. at 15°, about 1.871; B.p., 110.3°).
BENZENE (Sp. Gr. at 0°, 0.899, at 20°, 0.8799; B.p., 80.4°; M.p. + 5.4°).

5. SPECIAL TEST SOLUTIONS

BARFOED'S REAGENT.—*One part neutral cupric acetate added to 15 parts of water. Add to 200 c.c. of this solution 5 c.c. of 38 per cent. acetic acid.*

BENEDICT'S REAGENT, QUALITATIVE.—*Heat 173 grms. of sodium citrate and 100 grms. of anhydrous sodium carbonate with about 600 c.c. of water until dissolved. Filter and transfer filtrate to a litre flask, washing out the filter paper and flask, which originally contained the filtrate repeatedly with water (about 150 c.c. in all), and transferring the washings to the litre flask. Dissolve 17.3 grms. of crystalline copper sulphate in 100 c.c. of water in a beaker, and gradually add the copper sulphate solution to the alkaline citrate solution, shaking well after each addition. Wash out the beaker repeatedly with water, using the water to make up the contents of the volumetric flask to 1 litre. The chief advantages of Benedict's solution as compared with that of Fehling are:—(1) It does not deteriorate on standing. (2) It does not destroy small quantities of glucose, being less alkaline than Fehling's solution. (3) It is not reduced by chloroform.*

BRUCKE'S REAGENT.—*With the aid of heat prepare a saturated solution of mercuric iodide in 5 per cent. to 10 per cent. solution of potassium iodide.*

ESBACH'S REAGENT.—*Picric acid, 10 grms.; citric acid, 20 grms.; water to 1 litre.*

FEHLING'S SOLUTION.

1. **CUPRIC SULPHATE SOLUTION.**—*34.64 grms. of crystals of pure cupric sulphate dissolved in 500 c.c. of water.*

2. **ALKALINE TARTRATE SOLUTION.**—*173 grms. of sodium potassium tartrate, and 60 grms. of sodium hydrate dissolved in water and solution made up to 500 c.c. One vol. of (1) plus 1 vol. of (2) = Fehling's solution.*

I—CONTINUED

PAVY - FEHLING.—120 c.c. of Fehling's solution plus 300 c.c. of ammonia (sp. gr. .880) made up to a litre with water.¹

FURFURAL (SATURATED AQUEOUS SOLUTION).

GLYOXYLIC ACID SOLUTION (HOPKINS & COLE).—To 500 c.c. of a saturated solution of oxalic acid placed in a tall cylinder, add 30 grms. of sodium amalgam. When all the hydrogen has been evolved, filter and dilute the solution with twice or thrice its volume of water.

Benedict's method of preparation.—Place 10 grms. of magnesium in a large flask (at least 750 c.c.), add sufficient water to cover the magnesium completely, and then add slowly 250 c.c. of a cold saturated solution of oxalic acid. Cool the flask under the tap from time to time during the addition of the oxalic acid. When all the acid has been added, shake up thoroughly and filter off the precipitate of magnesium oxalate. Transfer the filtrate to a litre volumetric flask, wash the precipitate of magnesium oxalate and transfer the wash water to the litre flask. Acidify the contents of the litre flask with acetic acid, and make up to a litre with water. This solution contains the magnesium salt of glyoxylic acid.

MILLON'S REAGENT.—Dissolve with the aid of heat 1 part of mercury in two parts of concentrated nitric acid, and dilute the solution with 2 volumes of water.

NYLANDER'S SOLUTION.—100 grms. of caustic soda of sp. gr. 1.12, 4 grms. of sodium potassium tartrate, and 2 grms. of bismuth subnitrate.

PHENYLHYDRAZINE.

PHENYLHYDRAZINE HYDROCHLORIDE.

UFFELMANN'S REAGENT.—Add a few drops of ferric chloride to a 2 per cent. solution of carbolic acid.

6. INDICATORS

ALIZARIN RED S. (sodium sulphalizarate).—Shake up 1 gm. of the substance with 100 c.c. of water and filter. pH 0 -- 4, yellow to green, pH 5, brown, pH 6 -- 11, red, pH 12 -- 14, violet.

COCHINEAL.—Extract 5 grms. of cochineal with 150 c.c. alcohol, and 100 c.c. water, for several days. Then filter.

CONGO RED.—Dissolve 0.5 gm. of the sodium salt in 90 c.c. water and 10 c.c. alcohol (2 drops of solution to be titrated). pH 3, blue, pH 4, violet, and pH 5, scarlet.

DIMETHYLAMINO - AZOBENZENE. —Dissolve .05 gm. in 100 c.c. alcohol. pH 1 -- 2.5, crimson, pH 2.5 -- 3.5, flesh-coloured, and pH 3.5 → yellow (2 to 5 drops to 10 c.c.).

¹ The following is an alternative method:—4.1589 CuSO₄, 20.49 sodium potassium tartrate, 20.49 KOH, 300 c.c. ammonia (sp. gr. .880), and sufficient water to make the whole up to 1 litre.

I—CONTINUED

LITMUS SOLUTION.—Extract 10 grms. of finely powdered litmus with 20 c.c. of hot distilled water. Decant the clear blue solution from the sediment which forms on standing.

METHYL - ORANGE.—Dissolve 0.5 gm. of the solid in 500 c.c. of distilled water (or 0.1 gm. per litre (Sørensen)). Use 3 to 5 drops of the latter solution for each 10 c.c. of the solution to be titrated, pH4 (transition point).

METHYL - RED.—0.02 to 0.1 gm. dissolved in 30 per cent. alcohol (about 5 to 10 drops of former solution to 10 c.c.). pH4.4 -- 6.0, red-yellow.

METHYL - VIOLET (0.5 to .1 per cent. in aqueous solution).

NEUTRAL RED.—Dissolve .02 gm. in 100 c.c. alcohol, and 100 c.c. water (10 to 20 drops for 10 c.c.). Range pH6.8 to 8. Blue \rightarrow pH -- 0.3, blue, pH -- 0.3 to 0, blue-violet, pH6.8 to 8, red to orange, pH8 \rightarrow yellow.

α - NAPHTHOLPHTHALEIN. — Dissolve 0.1 gm. in 150 c.c. alcohol and 100 c.c. water (4 to 12 drops for 10 c.c.). pH7, reddish, pH8, greenish, pH9, blue.

P - NITROPHENOL.—0.2 per cent. alcoholic solution (about 2 to 4 drops for 10 c.c.). pH range 5 to 7.

PHENACETOLIN.—0.2 per cent. in alcohol. Range pH5 to 6. 0 to 3, yellow, 3 to 4, yellow to brown, 5 to 6, brown-red to pink, 6 to 10, pink, 11 to 12, violet, and 12 to 14, colourless.

PHENOLPHTHALEIN.—Dissolve 0.1 to 1 gm. of the solid in 100 c.c. of alcohol. Range 8.4 to 9.2 (Noyes), 8.3 to 10 (Sørensen) varies with concentration (3 to 20 drops of .05 per cent. solution for 10 c.c.).

PHLOROGLUCINOL AND VANILLIN (GÜNZBURG'S REAGENT).—Dissolve 2 grms. phloroglucinol and 1 gm. vanillin in 30 grms. of absolute alcohol.

ROSOLIC ACID.—Dissolve 1 gm. of the solid in 200 c.c. of alcohol (1 to 2 drops for 10 c.c.), pH6 (brownish), pH7 (pink), and pH8 (red).

THYMOLPHTHALEIN.—0.5 per cent. alcoholic solution (about 1 to 2 drops for 10 c.c.). Range 9.3 to 10.5 (Sørensen). Colour change is colourless to blue.

TROPÆOLIN OO (.05 to .1 per cent. in aqueous solution).

CLARK AND LUBS' INDICATORS.—The stock solutions contain 0.4 per cent. of the dyes. For use in titrations prepare by dilution 0.04 per cent. solutions of thymol blue, brom-thymol blue, brom-phenol blue, and brom-cresol purple, and 0.02 per cent. solutions of cresol-red, phenol-red, and methyl-red. Use 5 drops of the dilute solution of the dye for each 10 c.c. of the solution to be tested.

I—CONTINUED

7. CLARK AND LUBS' STANDARD pH SOLUTIONS AT 20° C.

KCl—HCl MIXTURES.¹

pH						
1.2	50 c.c.	$\frac{m}{5}$ KCl	.	.	64.5 c.c. $\frac{m}{5}$ HCl.	Dilute to 200 c.c.
1.4	"	"	.	.	41.5 " "	"
1.6	"	"	.	.	26.3 " "	"
1.8	"	"	.	.	16.6 " "	"
2.0	"	"	.	.	10.6 " "	"
2.2	"	"	.	.	6.7 " "	"

PHTHALATE—HCl MIXTURES.

2.2	50 c.c.	$\frac{m}{5}$ KH Phthalate,	46.70 c.c.	$\frac{m}{5}$ HCl.	Dilute to 200 c.c.
2.4	"	"	39.60	" "	"
2.6	"	"	32.95	" "	"
2.8	"	"	26.42	" "	"
3.0	"	"	20.32	" "	"
3.2	"	"	14.70	" "	"
3.4	"	"	9.90	" "	"
3.6	"	"	5.97	" "	"
3.8	"	"	2.63	" "	"

PHTHALATE—NaOH MIXTURES.

pH					
4.0	50 c.c.	$\frac{m}{5}$ KH Phthalate,	0.40 c.c.	$\frac{m}{5}$ NaOH.	Dilute to 200 c.c.
4.2	"	"	3.70	" "	"
4.4	"	"	7.50	" "	"
4.6	"	"	12.15	" "	"
4.8	"	"	17.70	" "	"
5.0	"	"	23.85	" "	"
5.2	"	"	29.95	" "	"
5.4	"	"	35.45	" "	"
5.6	"	"	39.85	" "	"
5.8	"	"	43.00	" "	"
6.0	"	"	45.45	" "	"
6.2	"	"	47.00	" "	"

Preparation of $\frac{m}{5}$ Phthalate Solutions.—Dissolve 60 grms. KOH in about 400 c.c. water. Add 50 grms. resublimed commercial ortho-phthalic acid anhydride. Test cool portion of solution with phenolphthalein, if alkaline add more phthalic anhydride, if acid more KOH. A diluted portion should give slight pink with phenolphthalein. Now

¹ pH values of this series given from preliminary measurements.

I—CONTINUED

add as much more phthalic anhydride as the solution contains, and heat until all is dissolved. Filter while hot, and allow crystallisation to take place slowly. Crystals are then drained by suction and recrystallised at least twice from distilled water. Dry salt at 110-115° C. to constant weight.

KH₂PO₄—NaOH MIXTURES.

pH	50 c.c. $\frac{m}{5}$ KH ₂ PO ₄ ,	3.72 c.c. $\frac{m}{5}$ NaOH. Dilute to 200 c.c.
5.8	"	"
6.0	"	"
6.2	"	"
6.4	"	"
6.6	"	"
6.8	"	"
7.0	"	"
7.2	"	"
7.4	"	"
7.6	"	"
7.8	"	"
8.0	"	"

Preparation of $\frac{m}{5}$ KH₂PO₄.—Take 27.232 grms. of the purest KH₂PO₄ (may be recrystallised thrice), dissolve in water and make up to one litre. The solution should be distinctly red with methyl red, and distinctly blue with brom-phenol blue.

BORIC ACID. KCl—NaOH MIXTURES.

pH	50 c.c. $\frac{m}{5}$ H ₃ BO ₃ , $\frac{m}{5}$ KCl,	2.61 c.c. $\frac{m}{5}$ NaOH. Dilute to 200 c.c.
7.8	"	"
8.0	"	"
8.2	"	"
8.4	"	"
8.6	"	"
8.8	"	"
9.0	"	"
9.2	"	"
9.4	"	"
9.6	"	"
9.8	"	"
10.0	"	"

Preparation of Boric-KCl Solution.—Take 12.4048 grms. boric acid (air-dried between filter paper over CaCl₂ in desiccator to constant weight) and 14.912 grms. purest KCl, dissolve in water, and make up to one litre.

For preparation of normal acid and alkali solutions, see p. 295.

II

REDUCTION OF ENGLISH TO METRICAL MEASURES OF CAPACITY, WEIGHT AND LENGTH

1. MEASURES OF CAPACITY

METRICAL SYSTEM

- 1 LITRE = 1 CUBIC DECIMETER (CBDM.) = 1000 CUBIC CENTIMETRES (CBCM. OR CC.). A CUBIC CENTIMETRE IS THE VOLUME OF 1 GRM. OF DISTILLED WATER AT 4° C.
1 CBCM. = 16.63 MINIMS.
1 LITRE = 35.2154 FLUID OUNCES.

ENGLISH SYSTEM

- 1 MINIM = .059 CBCM.
1 FLUID DRACHM = 3.549 C.C.
1 FLUID OUNCE = 28.398 C.C.
1 PINT = 567.936 C.C.

2. MEASURES OF WEIGHT

METRICAL SYSTEM

- 1 GRM. = THE WEIGHT OF 1 CBCM. OF WATER AT 4° C.
1 GRM. = 10 DECIGRMS. = 100 CENTIGRMS. = 1000 MILLIGRMS.
1 KILOGRAMME = 10 HEKTOGRMS. = 1000 GRMS.
1 GRM. = 15.43235 GRAINS.
1 KILOGRM. = 2 LBS. 3 OZ. 119.8 GRAINS.

ENGLISH SYSTEM

- 1 GRAIN = .0648 GRMS.
1 OUNCE (AVOIRDUPOIS) = 437.5 GRAINS = 28.3595 GRMS.

II—CONTINUED

3. MEASURES OF LENGTH

BRITISH AND METRIC EQUIVALENTS

1 INCH = 2.5400 CENTIMETRES (CM.)

1 CM. = 0.3937 INCH.

1 YARD = 0.9144 METRE.

1 METRE = 1.0936 YARD.

4. MEASURES OF TEMPERATURE

CONVERSION OF DEGREES F. INTO DEGREES C., AND *VICE VERSA*

$$t^{\circ} \text{ F.} = \frac{5}{9}(t - 32)^{\circ} \text{ C.}$$

$$t^{\circ} \text{ C.} = \frac{9}{5}t + 32^{\circ} \text{ F.}$$

III

ATOMIC WEIGHTS OF SOME OF THE ELEMENTS (O = 16)

Element.				Element.			
Aluminium	.	.	Al 27.1	Molybdenum	.	Mo 96.0	
Barium	.	.	Ba 137.37	Nickel	.	Ni 58.68	
Bismuth	.	.	Bi 208.0	Nitrogen	.	N 14.01	
Bromine	.	.	Br 79.92	Oxygen	.	O 16.0	
Calcium	.	.	Ca 40.07	Osmium	.	Os 190.9	
Carbon	.	.	C 12.005	Phosphorus	.	P 31.04	
Chlorine	.	.	Cl 35.46	Platinum	.	Pt 195.2	
Chromium	.	.	Cr 52.0	Potassium	.	K 39.10	
Copper	.	.	Cu 63.57	Radium	.	Ra 226.0	
Fluorine	.	.	F 19.0	Silicon	.	Si 28.3	
Hydrogen	.	.	H 1.008	Silver	.	Ag 107.88	
Gold	.	.	Au 197.2	Sodium	.	Na 23.00	
Iodine	.	.	I 126.92	Strontium	.	Sr 87.63	
Iron	.	.	Fe 55.84	Sulphur	.	S 32.06	
Lead	.	.	Pb 207.2	Tin	.	Sn 118.7	
Lithium	.	.	Li 6.94	Titanium	.	Ti 48.1	
Magnesium	.	.	Mg 24.32	Tungsten	.	W 184.0	
Manganese	.	.	Mn 54.93	Uranium	.	U 238.2	
Mercury	.	.	Hg 200.6	Zinc	.	Zn 65.37	

IV

WAVE LENGTHS OF THE CHIEF FRAUNHOFER LINES AND OF LINES IN THE EMISSION SPECTRA OF CERTAIN METALS

The wave lengths are expressed in millionths of a millimetre, i.e. .001 μ (or $\mu\mu$).

K (DOUBLE LINE)	769.9, 766.5
A (FRAUNHOFER'S LINE)	766.1
B (")	687
Li	670.8
C (FRAUNHOFER'S LINE)	656.3
Cd	643.9
a (FRAUNHOFER'S LINE)	627.8
Li (ORANGE YELLOW)	610.3
D (MIDDLE, FRAUNHOFER'S LINE)	589.3
Tl (GREEN)	535
E (FRAUNHOFER'S LINE)	527
b ₁ (")	518
F (")	486.1
Cd (BLUE)	480
Cd (")	467.8
Sr (")	460.7
d (FRAUNHOFER'S LINE)	438.3
G (")	430.8
K (VIOLET)	404.4
H (FRAUNHOFER'S LINE)	396.8

V

WEIGHTS OF CERTAIN MOLECULES
AND RADICLES

AgBr	187.8	Cr ₂ O ₃	152.0	H ₂ O	18.016
AgCl	143.34	CrO ₃	100.0	H ₃ PO ₄	98.06
AgCN	133.9	CuCNS	121.65	H ₂ PtCl ₆	410.0
AgI	234.8	Cu ₂ O	143.14	H ₂ S	34.08
AgNO ₃	169.89	CuO	79.57	H ₂ S ₂ O ₃	114.14
BaCO ₃	197.38	CuSO ₄	159.63	H ₂ SO ₃	82.08
BaCl ₂	208.29	CuSO ₄ ·5H ₂ O	249.71	H ₂ SO ₄	98.08
BaCl ₂ ·2H ₂ O	244.32	FeCl ₂ ·4H ₂ O	198.82	HgCl	236.1
Ba(NO ₃) ₂	261.39	FeCl ₃	162.22	HgCl ₂	271.5
BaO	153.37	FeO	71.84	KBr	119.02
Ba(OH) ₂ ·		Fe ₂ O ₃	159.68	KCN	65.12
8H ₂ O	315.51	FeSO ₄ ·7H ₂ O	278.01	KCNS	97.18
BaSO ₄	233.43	HBr	80.93	K ₂ CO ₃	138.21
Bi ₂ O ₃	464.0	HCN	27.02	KCl	74.56
CH ₂	14.021	HCNS	59.08	K ₂ Cr ₂ O ₄	194.2
CH ₃	15.029	H ₂ C ₂ O ₄ ·		K ₂ Cr ₂ O ₇	294.2
CH ₄	16.037	2H ₂ O	126.058	KI	166.02
C ₂ H ₅ OH	46.058	H ₂ ·C ₄ H ₄ O ₆	150.068	KIO ₃	214.02
C ₂ H ₃ O ₂	59.034	(Tartaric)		KMnO ₄	158.03
C ₆ H ₅	77.07	H ₃ ·C ₆ H ₅ O ₇ ·		KNO ₂	85.11
CO ₂	44.005	H ₂ O	210.11	KNO ₃	101.11
C ₂ O ₄	88.01	(Citric)		KOH	56.11
CO ₃	60.005	HCl	36.47	K ₂ PtCl ₆	486.2
CaC ₂ O ₄ ·H ₂ O	146.1	H ₃ Fe(CN) ₆	214.95	K ₂ SO ₄	174.26
CaCO ₃	100.08	H ₄ Fe(CN) ₆	215.96	MgCO ₃	84.33
CaCl ₂	110.99	HI	127.93	MgCl ₂ ·6H ₂ O	203.34
CaO	56.07	HNO ₃	63.02	MgO	40.32
CIO ₃	83.46	HO	17.008	Mg ₂ P ₂ O ₇	222.72

V—CONTINUED

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	246.49	$\text{Na}_2\text{CO}_3 \cdot$		PO_4	95.04
MnO	70.93	$10\text{H}_2\text{O}$	286.17	$\text{P}_2\text{O}_5 \cdot 24\text{MoO}_3$	3598
MnO_2	86.93	$\text{Na}_2\text{C}_2\text{O}_4 \cdot$		$\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot$	
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	241.07	H_2O	152.03	$3\text{H}_2\text{O}$	379.32
MoO_3	144.0	NaCl	58.46	PbCO_3	267.21
NH_3	17.03	NaF	42.0	PbCl_2	278.12
NH_4CNS	76.12	NaHCO_3	84.01	PbO	223.20
$(\text{NH}_4)_2\text{C}_2\text{O}_4 \cdot$		$\text{Na}_2\text{HPO}_4 \cdot$		PbS	239.26
H_2O	142.11	$12\text{H}_2\text{O}$	358.24	PbSO_4	303.26
NH_4Cl	53.50	NaHSO_3	104.07	PtCl_4	337.0
$\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot$		NaNO_2	69.01	SO_2	64.06
$12\text{H}_2\text{O}$	482.19	NaNO_3	85.01	SO_3	80.06
$\text{NH}_4\text{NaHPO}_4 \cdot$		Na_2O_2	78.0	SO_4	96.06
$4\text{H}_2\text{O}$	209.15	NaOH	40.01	S_2O_8	192.12
$(\text{NH}_4)_2\text{SO}_4$	132.14	NaPO_3	102.04	SnCl_2	189.6
$(\text{NH}_4)_2\text{Fe}$		$\text{Na}_2\text{S}_2\text{O}_8 \cdot$		SnCl_4	260.5
$(\text{SO}_4)_2 \cdot$		$5\text{H}_2\text{O}$	248.2	SnO	134.7
$6\text{H}_2\text{O}$	392.14	$\text{Na}_2\text{SO}_3 \cdot$		UO_2	270.5
NO	30.01	$7\text{H}_2\text{O}$	252.17	WO_3	232.0
N_2O_3	76.02	$\text{Na}_2\text{SO}_4 \cdot$		ZnCO_3	125.38
NO_2	46.01	$10\text{H}_2\text{O}$	322.22	$\text{ZnCl}_2 \cdot$	136.29
$\text{NaC}_2\text{H}_3\text{O}_2 \cdot$		$\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$	280.85	ZnO	81.37
$3\text{H}_2\text{O}$	136.08	P_2O_5	142.08	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	287.54
Na_2CO_3	106.01	P_2O_7	174.08		

VI

RELATION BETWEEN VOLUME AND WEIGHT (FOR CALIBRATION OF GLASS VESSELS)

[Kohlrausch and Holborn].

A clean dry glass vessel is weighed at temperature t° . It is then filled to a fixed mark with water or mercury and again weighed. The difference between the latter weight and the former weight gives the amount in grammes of water (wg.) or mercury (mg.) contained in the vessel at temperature t° . The volume of the vessel at 18° is then $w(1 + \delta)$ c.c. or mk c.c. The values of δ and k at different temperatures are given in the following table. The volume of the vessel at temperatures other than 18° may be calculated from the formula $v_t = v_{18} (1 + \frac{t - 18}{40,000})$, the volume expansion coefficient of glass being taken as 0.000025.

t°	δ	k	t°	δ	k
5	0.00140	0.073641	16	0.00214	0.073767
6	140	652	17	229	779
7	141	664	18	244	790
8	143	675	19	260	802
9	147	687	20	278	813
10	153	698	21	297	825
11	160	710	22	317	836
12	169	721	23	338	848
13	178	733	24	360	860
14	189	744	25	383	871
15	201	756	26	407	882

VII

TABLES OF THE SPECIFIC GRAVITY OF ALKALIES AND ACIDS AT 15° (Lunge) COMPARED WITH DISTILLED WATER AT 4° FOR 2. AND 3. AND AT 15° FOR 1.

Percentage signifies percentage by weight, or grms. of solute in 100 grms. of solution.

1.

Specific Gravity 15° 15°	Percent- age of	100 c.c. contain grms.	Specific Gravity.	Percent- age of	100 c.c. contain grms.	Specific Gravity.	Percent- age of	100 c.c. contain grms.
	NaOH.			KOH.			NH ₃ .	
1.014	1.20	1.2	1.014	1.7	1.7	0.998	0.45	0.45
1.022	2.00	2.1	1.029	3.5	3.6	0.994	1.37	1.36
1.036	3.35	3.5	1.045	5.6	5.8	0.990	2.31	2.29
1.045	4.00	4.2	1.060	7.4	7.8	0.986	3.30	3.25
1.052	4.64	4.9	1.075	9.2	9.9	0.982	4.30	4.22
1.060	5.29	5.6	1.091	10.9	11.9	0.978	5.30	5.18
1.075	6.55	7.0	1.100	12.0	13.2	0.974	6.30	6.14
1.091	8.00	8.7	1.116	13.8	15.3	0.970	7.31	7.09
1.100	8.68	9.5	1.134	15.7	17.8	0.966	8.33	8.05
1.116	10.06	11.2	1.152	17.6	20.3	0.962	9.35	8.99
1.134	11.84	13.4	1.171	19.5	22.8	0.958	10.47	10.03
1.152	13.55	15.6	1.190	21.4	25.5	0.954	11.60	11.07
1.171	15.13	17.7	1.210	23.3	28.2	0.950	12.74	12.10
1.190	16.77	20.0	1.231	25.1	30.9	0.946	13.88	13.13
1.210	18.58	22.5	1.252	27.0	33.8	0.942	15.04	14.17
1.231	20.59	25.3	1.274	28.9	36.8	0.938	16.22	15.21
1.252	22.64	28.3	1.297	30.7	39.8	0.934	17.42	16.27
1.274	24.81	31.6	1.320	32.7	43.2	0.930	18.64	17.34
1.297	26.83	34.8	1.345	34.9	46.9	0.926	19.87	18.42
1.320	28.83	38.1	1.370	36.9	50.6	0.922	21.12	19.47
1.345	31.22	42.0	1.397	38.9	54.3	0.918	22.39	20.56
1.370	33.69	46.2	1.424	40.9	58.2	0.914	23.68	21.63
1.397	36.25	50.6	1.453	43.4	63.1	0.910	24.99	22.74
1.424	38.80	55.3	1.483	45.8	67.9	0.906	26.31	23.83
1.453	41.41	60.2	1.514	48.3	73.1	0.902	27.65	24.94
1.483	44.38	65.8	1.546	50.6	77.9	0.898	29.01	26.05
1.514	47.60	72.1	1.580	53.2	84.0	0.894	30.37	27.15
...	1.615	55.9	90.2	0.890	31.75	28.26
...	1.634	57.5	94.0	0.886	33.25	29.46
..	0.882	34.95	30.83

VII—CONTINUED

2.

Specific Gravity $\frac{15^{\circ}}{4^{\circ}}$	Percent- age of	100 C.C. contain grms.	Specific Gravity $\frac{15^{\circ}}{4^{\circ}}$	Percent- age of	100 C.C. contain grms.	Specific Gravity $\frac{15^{\circ}}{4^{\circ}}$	Percent- age of	100 C.C. contain grms.
1.010	2.14	2.2	1.020	3.03	3.1	1.020	3.70	3.8
1.015	3.12	3.2	1.040	5.96	6.2	1.030	5.50	5.7
1.020	4.13	4.2	1.060	8.77	9.3	1.040	7.26	7.5
1.025	5.15	5.3	1.080	11.60	12.5	1.050	8.99	9.4
1.030	6.15	6.4	1.100	14.35	15.8	1.060	10.68	11.3
1.035	7.15	7.4	1.120	17.01	19.1	1.070	12.33	13.2
1.040	8.16	8.5	1.140	19.61	22.3	1.080	13.95	15.1
1.045	9.16	9.6	1.160	22.19	25.7	1.090	15.53	16.9
1.050	10.17	10.7	1.180	24.76	29.2	1.100	17.11	18.8
1.055	11.18	11.8	1.200	27.32	32.8	1.110	18.67	20.7
1.060	12.19	12.9	1.220	29.84	36.4	1.120	20.23	22.7
1.065	13.19	14.1	1.240	32.28	40.0	1.130	21.77	24.6
1.070	14.17	15.2	1.260	34.57	43.5	1.140	23.31	26.6
1.075	15.16	16.3	1.280	36.87	47.2	1.150	24.84	28.6
1.080	16.15	17.4	1.300	39.19	51.0	1.160	26.36	30.6
1.085	17.13	18.6	1.320	41.50	54.8	1.170	27.88	32.6
1.090	18.11	19.7	1.340	43.74	58.6	1.180	29.38	34.7
1.095	19.06	20.9	1.360	45.88	62.4	1.190	30.88	36.7
1.100	20.01	22.0	1.380	48.00	66.2	1.200	32.36	38.8
1.105	20.97	23.2	1.400	50.11	70.2	1.210	33.82	40.9
1.110	21.92	24.3	1.420	52.15	74.0	1.220	35.28	43.0
1.115	22.86	25.5	1.440	54.07	77.9	1.230	36.78	45.2
1.120	23.82	26.7	1.460	55.97	81.7	1.240	38.29	47.5
1.125	24.78	27.8	1.480	57.83	85.6	1.250	39.82	49.8
1.130	25.75	29.1	1.500	59.70	89.6	1.260	41.34	52.1
1.135	26.70	30.3	1.520	61.59	93.6	1.270	42.87	54.4
1.140	27.66	31.5	1.540	63.43	97.7	1.280	44.41	56.8
1.145	28.61	32.8	1.560	65.08	101.5	1.290	45.95	59.3
1.150	29.57	34.0	1.580	66.71	105.4	1.300	47.49	61.7
1.155	30.55	35.3	1.600	68.51	109.6	1.310	49.07	64.3
1.160	31.52	36.6	1.620	70.32	113.9	1.320	50.71	66.9
1.165	32.49	37.9	1.640	71.99	118.1	1.330	52.37	69.7
1.170	33.46	39.2	1.660	73.64	122.2	1.340	54.07	72.5
1.175	34.42	40.4	1.680	75.42	126.7	1.350	55.79	75.3
1.180	35.39	41.8	1.700	77.17	131.2	1.360	57.57	78.3
1.185	36.31	43.0	1.720	78.92	135.7	1.370	59.39	81.4
1.190	37.23	44.3	1.740	80.68	140.4	1.380	61.27	84.6
1.195	38.16	45.6	1.760	82.44	145.1	1.390	63.23	87.9

VII—CONTINUED

Specific Gravity $\frac{15^{\circ}}{4^{\circ}}$	Percent- age of	100 C.C. contain grms.	Specific Gravity $\frac{15^{\circ}}{4^{\circ}}$	Percent- age of	100 C.C. contain grms.	Specific Gravity $\frac{15^{\circ}}{4^{\circ}}$	Percent- age of	100 C.C. contain grms.
	HCl.			H ₂ SO ₄ .			HNO ₃ .	
1.200	39.11	46.9	1.780	84.50	150.4	1.400	65.30	91.4
...	1.800	86.90	156.4	1.410	67.50	95.2
...	1.820	90.05	163.9	1.420	69.80	99.1
...	1.840	95.60	175.9	1.430	72.17	103.2
...	1.8405	95.95	176.5	1.440	74.68	107.5
...	1.8415	97.70	179.9	1.450	77.28	112.1
...	1.8405	98.70	181.6	1.460	79.98	116.8
...	1.8400	99.20	182.5	1.470	82.90	121.9
...	1.480	86.05	127.4
...	1.490	89.60	133.5

3.

Specific gravity of phosphoric acid solutions compared with that of distilled water at 4°.

100 parts by weight of phosphoric acid solution contain parts by weight.

At 15°.

Sp. gr.	P ₂ O ₅ .	H ₃ PO ₄ .	Sp. gr.	P ₂ O ₅ .	H ₃ PO ₄ .
1.0054	0.726	1.0	1.1889	21.780	30.0
1.0109	1.452	2.0	1.2262	25.410	35.0
1.0164	2.178	3.0	1.2651	29.040	40.0
1.0276	3.630	5.0	1.3059	32.670	45.0
1.0567	7.260	10.0	1.3486	36.300	50.0
1.0874	10.890	15.0	1.3931	40.030	55.0
1.1196	14.520	20.0	1.4395	43.560	60.0
1.1534	18.150	25.0			

At 17.5°.

Sp. gr.	Percentage H ₃ PO ₄ .	Sp. gr.	Percentage H ₃ PO ₄ .	Sp. gr.	Percentage H ₃ PO ₄ .
1.809	93.67	1.701	84.72	1.536	70.26
1.800	92.99	1.677	82.65	1.513	68.19
1.792	92.30	1.653	80.59	1.491	66.12
1.783	91.61	1.629	78.52	1.469	64.06
1.775	90.92	1.605	76.45	1.448	61.99
1.750	88.85	1.581	74.39	1.428	59.92
1.725	86.79	1.559	72.32	1.409	57.86

VIII

SPECIFIC GRAVITY AND PERCENTAGE BY WEIGHT OF AQUEOUS ALCOHOL AT 15.5° (Fownes)

Specific Gravity.	Per Cent.	Specific Gravity.	Per Cent.	Specific Gravity.	Per Cent.
0.9184	50	0.8793	67	0.8382	84
0.9160	51	0.8769	68	0.8357	85
0.9135	52	0.8745	69	0.8331	86
0.9113	53	0.8721	70	0.8305	87
0.9090	54	0.8696	71	0.8279	88
0.9069	55	0.8672	72	0.8254	89
0.9047	56	0.8649	73	0.8228	90
0.9025	57	0.8625	74	0.8199	91
0.9001	58	0.8603	75	0.8172	92
0.8979	59	0.8581	76	0.8145	93
0.8956	60	0.8557	77	0.8118	94
0.8932	61	0.8533	78	0.8089	95
0.8908	62	0.8508	79	0.8061	96
0.8886	63	0.8483	80	0.8031	97
0.8863	64	0.8459	81	0.8001	98
0.8840	65	0.8434	82	0.7969	99
0.8816	66	0.8408	83	0.7938	100

IX

SOLUBILITIES OF SOME SALTS IN 100 PARTS OF
WATER AT DIFFERENT TEMPERATURES (*t*)

<i>t</i> .	Parts (NH ₄) ₂ SO ₄ .	MgSO ₄ .	Na ₂ SO ₄ .	Na ₂ SO ₄ + 10H ₂ O.	ZnSO ₄ .	ZnSO ₄ + 7H ₂ O.	NaCl.
0	70.6	26.9	5.02	12.17	43.02	115.2	35.7
5	71.8	29.3	6.4	35.7
10	73	31.5	9	...	48.36	138.2	35.8
15	74.2	33.8	12.49	35.49	35.9
20	75.4	36.2	19.5	...	53.13	161.5	36
25	76.7	38.5	28.1	99.4	36.1
30	78	40.9	40.9	...	58.4	190.9	36.3
35	79.5	43.3	50.2	36.4
40	81	45.6	48.8	291.4	63.52	224	36.6
45	82.7	48	47.7	36.8
50	84.4	50.3	46.7	...	68.75	263.8	37
55	86.2	52.7	45.9	37.1
60	88	55	45.3	...	74.2	313.5	37.3
65	89.9	57.3	44.8	37.5
70	91.6	59.6	44.4	...	79.25	369.3	37.9
75	93.4	61.9	44	38.2
80	95.3	64.2	43.7	...	84.6	442.6	38.4
85	97.2	66.5	43.3	38.7
90	99.2	68.9	43.1	...	89.78	533	39.1
95	101.2	71.4	42.8	39.4
100	103.3	73.8	42.5	...	95	653.6	39.8

Maximum solubility of sodium sulphate is at 33°. At 32.73°, 100 parts of water dissolve 50.65 parts of anhydrous sodium sulphate and 322.12 parts of Na₂SO₄ + 10H₂O.

X

SOLUBILITIES OF CERTAIN IMPORTANT SUBSTANCES IN WATER AT 15°

(Küster-Thiel. Rechentafeln für Chemiker, p. 46.)

Substance.	Formula and molecular weight.	Per cent. ¹	Anhydrous.	Mols. per litre.	Sp. gr.
Ammonium chloride	NH_4Cl . (53.5)	26		5.22	1.073
„ oxalate	$(\text{NH}_4)_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$. (142.1)	4.22	3.69	0.302	1.017
„ sulphate	$(\text{NH}_4)_2\text{SO}_4$. (132)	42.6		4.01	1.244
Barium chloride	$\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$. (244)	31	25.6	1.57	1.277
„ hydroxide	$\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$. (315)	5.41	2.94	0.177	1.030
Calcium chloride	$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$. (219)	80.9	41.0	5.22	1.413
Cupric sulphate	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. (249.7)	25.34	16.20	1.203	1.185
Ferric ammonium sulphate	$\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$. (482.2)	42.94	23.66	1.083	1.217
Ferrous ammonium sulphate	$(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$. (392)	27.23	19.72	0.811	1.168
Ferric chloride	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. (270.2)	77.3	46.4	4.31	1.508
Ferrous chloride	$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$. (199)	63.1	40.2	4.66	1.47
„ sulphate	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. (278)	35.42	19.36	1.539	1.208
Magnesium sulphate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. (246.5)	51.0	24.9	2.65	1.281
Mercuric chloride	HgCl_2 . (271.5)	6.54		0.254	1.056
Oxalic acid	$\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$. (126.058)	10.8	7.7	0.89	1.038

¹ Percentage signifies grammes of solute present in 100 grammes solution.

X—CONTINUED

Substance.	Formula and molecular weight.	Per cent. ¹	Anhydrous.	Mols. per litre.	Sp. gr.
Potassium bichromate	$K_2Cr_2O_7$ (294.2)	8.34		0.301	1.062
„ bromide	KBr. (119)	38.9		4.42	1.351
„ chlorate	$KClO_3$ (122.56)	5.79		0.490	1.037
„ chloride	KCl. (74.56)	24.4		3.82	1.167
„ chromate	K_2CrO_4 (194.2)	38.2		2.71	1.380
„ ferrocyanide	$K_4Fe(CN)_6 \cdot 3H_2O$ (422.4)	20.6	18.0	0.558	1.141
„ iodide	KI. (166.02)	58.4		6.00	1.705
„ permanganate	$KMnO_4$ (158.03)	4.95		0.325	1.036
„ sulphate	K_2SO_4 (174.26)	9.25		0.571	1.076
Sodium acetate	$NaC_2H_3O_2 \cdot 3H_2O$ (136.1)	50.3	30.3	4.28	1.158
„ carbonate	$Na_2CO_3 \cdot 10H_2O$ (286.17)	37.8	14.0	1.52	1.148
„ chloride	NaCl. (58.46)	26.35		5.422	1.203
Disodium phosphate	$Na_2HPO_4 \cdot 12H_2O$ (358.24)	15.0	5.95	0.444	1.060
Sodium sulphate	$Na_2SO_4 \cdot 10H_2O$ (322.2)	26.54	11.70	0.913	1.108
„ thiosulphate	$Na_2S_2O_3 \cdot 5H_2O$ (248.2)	62.0	39.5	3.44	1.377
Silver nitrate	$AgNO_3$ (169.89)	64.9		7.83	2.05
Stannous chloride	$SnCl_2 \cdot 2H_2O$ (225.7)	86.9	73.0	8.03	2.09
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$ (287.54)	60.03	33.70	3.01	1.44

¹ Percentage signifies grammes of solute present in 100 grammes solution.

XI

SPECIFIC GRAVITY ($\frac{15}{4}$) AND NORMALITY OF CERTAIN SOLUTIONS (Küster)

Sp. gr. $\frac{15}{4}$	Normality of						Sp. gr. $\frac{15}{4}$	Normal NH ₃ .
	H ₂ SO ₄ .	HCl.	HNO ₃ .	KOH.	NaOH.	Na ₂ CO ₃ .		
1.010	0.324	0.593	0.305	0.213	0.239	0.198	0.995	0.666
1.020	0.634	1.155	0.599	0.413	0.464	0.383	0.990	1.224
1.030	0.951	1.737	0.899	0.616	0.700	0.571	0.985	1.934
1.040	1.264	2.328	1.197	0.822	0.939	0.762	0.980	2.637
1.050	1.578	2.929	1.497	1.032	1.182	0.956	0.975	3.343
1.060	1.896	3.544	1.796	1.246	1.431	1.153	0.970	4.043
1.070	2.223	4.158	2.092	1.462	1.684	1.353	0.965	4.740
1.080	2.555	4.784	2.389	1.682	1.942	1.556	0.960	5.453
1.090	2.887	5.414	2.685	1.903	2.205	1.762	0.955	6.208
1.100	3.219	6.037	2.985	2.128	2.472	1.971	0.950	6.966
1.110	3.556	6.673	3.287	2.356	2.744	2.183	0.945	7.722
1.120	3.885	7.317	3.594	2.586	3.021	2.408	0.940	8.480
1.130	4.219	7.981	3.902	2.819	3.302	2.626	0.935	9.251
1.140	4.559	8.648	4.215	3.046	3.588	2.847	0.930	10.03
1.150	4.903	9.327	4.531	3.292	3.878	3.071	0.925	10.81
1.160	5.249	10.03	4.850	3.532	4.173	...	0.920	11.59
1.170	5.600	10.74	5.174	3.778	4.472	...	0.915	12.39
1.180	5.958	11.45	5.499	4.023	4.776	...	0.910	13.19
1.190	6.319	12.15	5.828	4.272	5.084	...	0.905	13.99
1.200	6.685	12.87	6.159	4.523	5.397	...	0.900	14.80
1.210	7.052	...	6.490	4.776	5.714	...	0.895	15.61
1.220	7.424	...	6.827	5.030	6.039	...	0.890	16.42

XI—CONTINUED

Sp. gr. $\frac{15}{4}$	Normality of						Sp. gr. $\frac{15}{4}$	Normal NH ₃ .
	H ₂ SO ₄ .	HCl.	HNO ₃ .	KOH.	NaOH.	Na ₂ CO ₃ .		
1.230	7.803	...	7.175	5.288	6.365	...	0.885	17.30
1.240	8.162	...	7.531	5.550	6.693	...	0.880	18.26
1.250	8.521	...	7.894	5.811	7.032
1.260	8.882	...	8.261	6.075	7.375
1.270	9.248	...	8.635	6.341	7.722
1.280	9.623	...	9.016	6.609	8.078
1.290	10.00	...	9.401	6.882	8.432
1.300	10.39	...	9.792	7.153	8.795
1.310	10.78	...	10.20	7.423	9.166
1.320	11.17	...	10.62	7.704	9.542
1.330	11.57	...	11.05	7.981	9.921
1.340	11.95	...	11.49	8.264	10.309
1.350	12.34	...	11.95	8.547	10.704

EXPLANATORY NOTE.

Intermediate values may be calculated from those given in the table by interpolation.

EXAMPLE.

The specific gravity of an aqueous solution of sulphuric acid at 15° was found to be 1.1530. One finds from the table that an acid of sp. gr. 1.1500 is 4.903 N and one of sp. gr. 1.1600 is 5.249 N. Hence an acid of sp. gr. 1.1530 is $4.903 + 30/100(5.249 - 4.903) = 5.007$ N.

XII

PREPARATION OF NORMAL SOLUTIONS

THE term "normal" as applied to solutions has already been defined (pp. 295 and 296).

Since oxalic acid ($\text{C}_2\text{H}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) can readily be obtained in a chemically pure state, N/1, N/2, N/5, and N/10 solutions of this acid form suitable starting-points for standardising carbonate free solutions of NaOH or KOH.

Solutions of oxalic acid, especially when dilute, *e.g.* N/10, become weaker on standing owing to oxidation of the acid. Further, oxalic acid is not a sufficiently strong acid to give a sharp end point with all acidimetric indicators. For these reasons oxalic acid should only be used in the initial preparation of standard NaOH or KOH solutions.

Preparation of a caustic soda solution (nearly free from carbonate).

Dissolve 100 grms. of pure caustic soda in 125 c.c. freshly distilled water in a resistance glass flask, and transfer when cooled to a cylinder closed with a rubber stopper (which has been previously freed from sulphur by heating with dilute caustic soda, and by finally repeatedly boiling with water). The carbonate settles to the bottom in about two days. Siphon off the supernatant solution and filter it into a resistance glass flask through a funnel loosely plugged with glass wool. This stock solution is about 17 N. Dilute with freshly distilled water (preferably "conductivity water") to about N/1 strength. Transfer at least 25 c.c. of this solution with a pipette into a flask, and titrate with N/1 oxalic acid, using phenolphthalein. From the result obtained calculate the dilution required for preparing N/1, N/5, and N/10 solutions of caustic soda. N/5 is a convenient concentration. It is advisable to dilute the original solution so as to obtain a solution very slightly stronger than N/5; 25 or more c.c. of the approximately N/5 solution are then titrated with N/5 oxalic acid. Then transfer the approximately N/5 solution to a volumetric flask (1000 or 2000 c.c.), and add from a burette the quantity of freshly distilled water required to bring it to N/5 strength. Store the solution thus obtained in a well-stoppered paraffined bottle.¹

¹ Thoroughly clean and dry a bottle, heat it in a drying oven to about 10° above the melting point of the paraffin. Pour in sufficient melted paraffin to form a fairly thick uniform coat. Roll the bottle gently so as to obtain an even coat, and stand the bottle upright just before the paraffin commences to solidify.

XII—CONTINUED

When diluting the solutions, avoid exposure to the air as far as possible, and use only freshly distilled water.

Preparation of normal and fractional normal solutions of hydrochloric acid.

Dilute chemically pure concentrated hydrochloric acid with an equal volume of distilled water and mix thoroughly. Determine the specific gravity of this solution with an accurate hydrometer, and ascertain from Table XI. the normality of the dilute acid. Using this value as a basis, calculate the degree of dilution which will be required to bring the acid to normal strength. Prepare 200 c.c. of approximately N/1 acid and determine its exact normality by titrating at least 25 c.c. with N/1 NaOH, using methyl red or phenolphthalein solution as indicator. With the aid of the latter result, prepare larger quantities of the N/1 acid (1 to 2 litres or more).

A normal solution of sulphuric acid may be prepared in a similar way. If desired, the strength of these acids may be checked gravimetrically by precipitation with silver nitrate solution in the one case and barium chloride solution in the other. The first method is obviously based on the assumption that the hydrochloric acid used is chemically pure and therefore free from non-volatile chlorides. The purity of the acid may be ensured by diluting the concentrated acid to a sp. gr. 1.010, and purifying by distillation. The acid may also be prepared from pure sodium chloride. The reader is referred to textbooks of volumetric analysis for fuller details.

Note on other methods of defining the concentrations of solutions.

(1) If a solution contain s grms. of solute in 100 grms. of solution, the percentage concentration by weight is 100 s . The majority of tables giving the relation between the specific gravities of solutions and concentrations of solutes refers to percentage concentrations by weight.

(2) If a solution contains s' grms of solute in 1 c.c. of solution at 15°, the percentage concentration by volume is 100 s' . The percentage content of a solution in physiological chemistry is usually defined in this way.

(3) When 1000 c.c. of a solution at 15° contain the molecular weight of the solute in grammes, the solution is said to be molar (M). M/2, M/5, M/10, M/15, and other fractional molar solutions are frequently used.

(4) A number of other methods are also used in defining the concentration of a solution. Thus in stating the solubilities of certain solutes at different temperatures, the concentration is sometimes defined as grammes of solute dissolved by 100 grammes of solvent.

XIII

TENSION OF AQUEOUS VAPOUR IN MM. OF MERCURY FROM 9° TO 20° C.

°	mm.	°	mm.	°	mm.	°	mm.	°	mm.
9.0	8.6	11.2	9.9	13.4	11.5	15.6	13.2	17.8	15.2
9.2	8.7	11.4	10.1	13.6	11.6	15.8	13.4	18.0	15.4
9.4	8.8	11.6	10.2	13.8	11.8	16.0	13.5	18.2	15.6
9.6	8.9	11.8	10.3	14.0	11.9	16.2	13.7	18.4	15.7
9.8	9.0	12.0	10.5	14.2	12.1	16.4	13.9	18.6	15.9
10.0	9.2	12.2	10.6	14.4	12.2	16.6	14.1	18.8	16.1
10.2	9.3	12.4	10.7	14.6	12.4	16.8	14.2	19.0	16.3
10.4	9.4	12.6	10.9	14.8	12.5	17.0	14.4	19.2	16.6
10.6	9.5	12.8	11.0	15.0	12.7	17.2	14.6	19.4	16.8
10.8	9.7	13.0	11.2	15.2	12.9	17.4	14.8	19.6	17.0
11.0	9.8	13.2	11.3	15.4	13.0	17.6	15.0	19.8	17.2
...	20.0	17.4

XIV

REDUCTION OF GASEOUS VOLUMES TO 0° AND 760 MM. OF Hg.

The corrected volume of the dry gas $V_0 = \frac{V(P - p)}{(1 + .00367t)760}$, where V , P , p , and t are the observed volume of the gas saturated with water vapour, barometric pressure, tension of aqueous vapour (in mm. of Hg), and observed temperature t° . The gas measured is assumed to be saturated with water vapour.

Values of $1 + .00367t$.

t°	0	1	2	3	4
0°	1.0000	1.0037	1.0073	1.0110	1.0147
5°	.0183	.0220	.0257	.0294	.0330
10°	.0367	.0404	.0440	.0477	.0514
15°	.0550	.0587	.0624	.0661	.0697
20°	.0734	.0771	.0807	.0844	.0881

Values of $\frac{P - p}{760}$

P-p.	0	1	2	3	4	5	6	7	8	9
710	.9342	.9355	.9368	.9382	.9395	.9408	.9421	.9434	.9447	.9461
720	.9474	.9487	.9500	.9513	.9526	.9539	.9553	.9566	.9579	.9592
730	.9605	.9618	.9632	.9645	.9658	.9671	.9684	.9697	.9711	.9724
740	.9737	.9750	.9763	.9776	.9789	.9803	.9816	.9829	.9842	.9855
750	.9868	.9882	.9895	.9908	.9921	.9934	.9947	.9961	.9974	.9987
760	1.0000	1.0013	1.0026	1.0039	1.0053	1.0066	1.0079	1.0092	1.0105	1.0118
770	1.0132	1.0145	1.0158	1.0171	1.0184	1.0197	1.0211	1.0224	1.0237	1.0250

Densities of gases.

One litre of hydrogen at 0° and 760 mm. weighs 0.08987 gram. The corresponding values for nitrogen, oxygen, carbon dioxide, and carbon monoxide are 1.2507, 1.4290, 1.9768, and 1.2504 gram.



SECTION IV .

SCHEMES FOR DETECTION OF
UNKNOWN SUBSTANCES
OF PHYSIOLOGICAL IMPORTANCE



EXAMINATION OF AN UNKNOWN SOLID OR MIXTURE OF SOLIDS OF PHYSIOLOGICAL IMPORTANCE

I. PHYSICAL CHARACTERS

NOTE the colour, odour, and taste. Place a small quantity on a slide and examine microscopically, and note whether crystalline or amorphous. Note the form (crystalline or amorphous), arrangement, etc., as, *e.g.* concentrically striated structure of starch granules, the notched plates of cholesterol, etc. The colour may suggest blood, bile, urinary pigments or lipochromes; the odour may suggest indole, skatole, phenol, mercaptans, etc.; a bitter taste may suggest proteoses, bile salts or acids, or creatinine; a sweet taste, a sugar of some kind, a polyhydric alcohol, or certain amino-acids. The majority of the proteins and the polysaccharides are practically tasteless.

II. EFFECT OF HEATING

Heat gradually over the open flame a small quantity of the finely powdered dry substance in a hard glass test-tube.

1. If it be a **non-nitrogenous** organic substance, then it will usually char, either with or without previous melting, and will frequently emit a more or less characteristic odour. Mono- and di-saccharides melt prior to charring, polysaccharides char without previous melting. The higher fatty acids decompose on heating and emit the odour of lower fatty acids. Mix part of the substance with anhydrous KHSO_4 and heat = odour of acrolein, if neutral fat or glyceride be present. If free fatty acids be present, the odour is of the lower volatile fatty acids. Phenol (see p. 132) melts at 42° and boils at 180° , emitting its characteristic odour. Cholesterol (see p. 149) melts at 145° .

2. If a **nitrogenous substance** be heated, either the odour or other physical and chemical characters of the products of decomposition may be distinctive. For example, proteins char on heating and yield the disagreeable odour of burnt horn. The behaviour of the following is more or less characteristic—(a) Urea (pp. 206, 207), (b) hippuric acid (p. 108), (c) uric acid (chars, yields some cyanuric acid and a very distinct odour of HCN), (d) tyrosine (p. 140, iv. (3)), (e) leucine (p. 140), (f) indole (pp. 130, 132), (g) skatole (pp. 130, 132), (h) glycine (p. 140), and (i) tryptophane. Tryptophane (i) becomes brown and melts at 252° . When heated to a higher temperature, it yields indole, skatole, and CO_2 . Test the vapours with a pinewood match dipped in concentrated hydrochloric acid (p. 131, 7). So far as time permits, test for the elements N, P, S

EXAMINATION OF AN UNKNOWN SOLID—CONTINUED

(loosely and firmly combined), and iodine. The results of this preliminary examination will frequently guide one as to the best method for proceeding further.

When proteins are present as a dry powder, their selective extraction by the methods described on p. 193 is frequently a tedious process, since they dissolve very slowly on account of their colloid nature. It is therefore advisable first to ascertain whether any proteins are present. This may be done in the following way:—

Extract the powder thoroughly with boiling water, and filter. If the filtrate give a distinct biuret reaction, proteoses, peptone, acid or alkali metaprotein (containing sufficient combined acid or base to render them soluble in water), salts of the mucins, nucleo- or phospho-proteins may be present. These may be partially identified by applying the methods given in Scheme II. Certain nucleo-proteins are partially decomposed by boiling water. If the filtrate do not give a biuret reaction, then the powder does not contain the above proteins; but the filtrate may obviously contain other substances of physiological importance, the examination of which will be described later.

Suspend a portion of the undissolved residue in Millon's reagent and heat. If the residue becomes red, coagulated protein is probably present. Dissolve another portion of the residue in hot dilute NaOH solution, cool, and add dilute CuSO_4 solution drop by drop. If biuret test be positive, coagulated protein is present. If negative, heat. A white precipitate indicates uric acid.

If both filtrate and residue, or residue alone contain protein, an attempt should be made to extract the proteins selectively from the original powder by the methods given on p. 193. Any protein which remains undissolved may be regarded as coagulated protein, *e.g.* fibrin, or protein coagulated by heat, collagen, or some plant protein such as gliadin.

Extract part of the original powder with 70 per cent. alcohol at 60°, following the method given on p. 94, 5. If this method give a positive result gliadin or another prolamine is present, and the powder is probably flour. Verify by the methods given on p. 94.

The undissolved residue may contain insoluble or sparingly soluble substances other than proteins, such as cholesterol, neutral fats, fatty acids, uric acid, calcium oxalate, or inorganic substances such as Ca, Mg, or ferric phosphates. If any of these be present, the effect of heating the dried mixture in a dry test-tube will probably give the worker sufficient indications to guide him in his subsequent procedure.

The following method (Scheme I.) may be used to give an indication of the class to which the body belongs, and from the results of the examination the worker may be enabled to proceed to one or other of the succeeding tables, according as he finds a carbohydrate, protein, or other substance (giving neither carbohydrate nor protein reactions) present.

SCHEME I.

EXTRACT SOME OF THE FINELY POWDERED SUBSTANCE WITH WATER AT 30° TO 40°. FILTER AND TEST FILTRATE AND RESIDUE. ANY NEARLY COLOURLESS SOLUTION MAY BE EXAMINED IN THE SAME WAY AS THE FILTRATE.

EXTRACT.

RESIDUE.

Test by Millon's, xantho-
proteic, or biuret reactions
for protein.

FILTRATE.

Render alkaline with NaOH¹;
add drop by drop dilute CuSO₄.

Blue pp. [Cu(OH)₂].

Heat.

Clear blue
solution.²

Heat.

Bluish-violet
(Gelatin).

Violet
(Native Protein).

Pink
(Proteoses or
Peptone).

Brownish-
black pp.
(negative
result).

White
then red
pp. of Cu₂O
(O.S.
Urate).

Pp. of CuOH
or Cu₂O
(Reducing
Carbo-
hydrate).

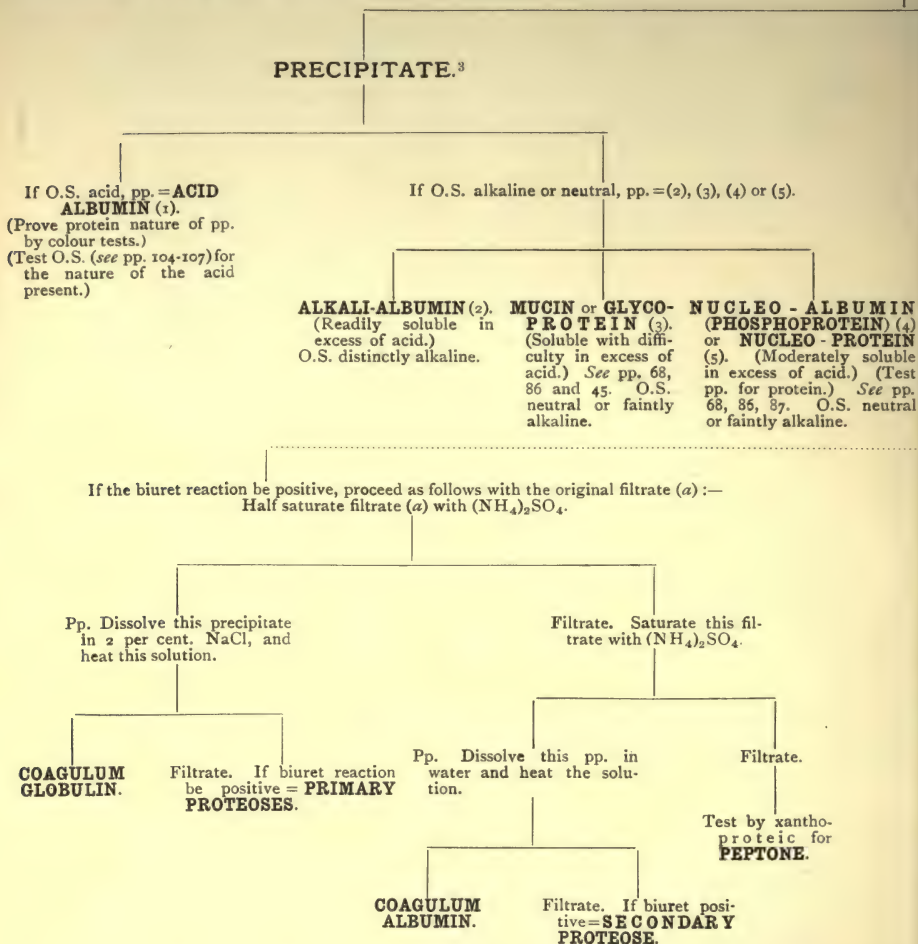
No Reduction,³
probably =
Glycerol, other
polyhydric alcohols,
or certain hydroxy
acids, *e.g.* Tartaric
or Citric Acid, or
Cane Sugar.

¹ Since sodium are less soluble than potassium soaps, a precipitate of sodium soaps may separate when NaOH solution is added to a solution of a potassium soap.

² If ammonium salts be present, a clear blue solution is also obtained. These are easily recognised by the detection of NH₃ set free by the addition of NaOH.

³ If protein is suspected, either in residue or filtrate, proceed to Scheme II.; if carbohydrate, proceed to Scheme III.; if neither protein nor carbohydrate, proceed to Scheme V.; if the solution be coloured or opaque, refer to Scheme IV.

TAKE REACTION OF SOLUTION. IF ACID OR ALKALINE, NEARLY CASE, OR RENDER FAINTLY ACID WITH ACETIC ACID IN THE LATTER. GLYCOPROTEIN, PHOSPHO- OR NUCLEO-PROTEIN, OR OF ANY PROTEIN RENDERED FAINTLY ACID WITH ACETIC ACID.



¹ Prepare aqueous, saline, and alkaline extracts (1—2 per cent. Na_2CO_3) of the powder (see pp. 92, 93) are given out for examination.

² Bile salts, if present, may be identified by the sulphur surface tension test (p. 148), or the solution may

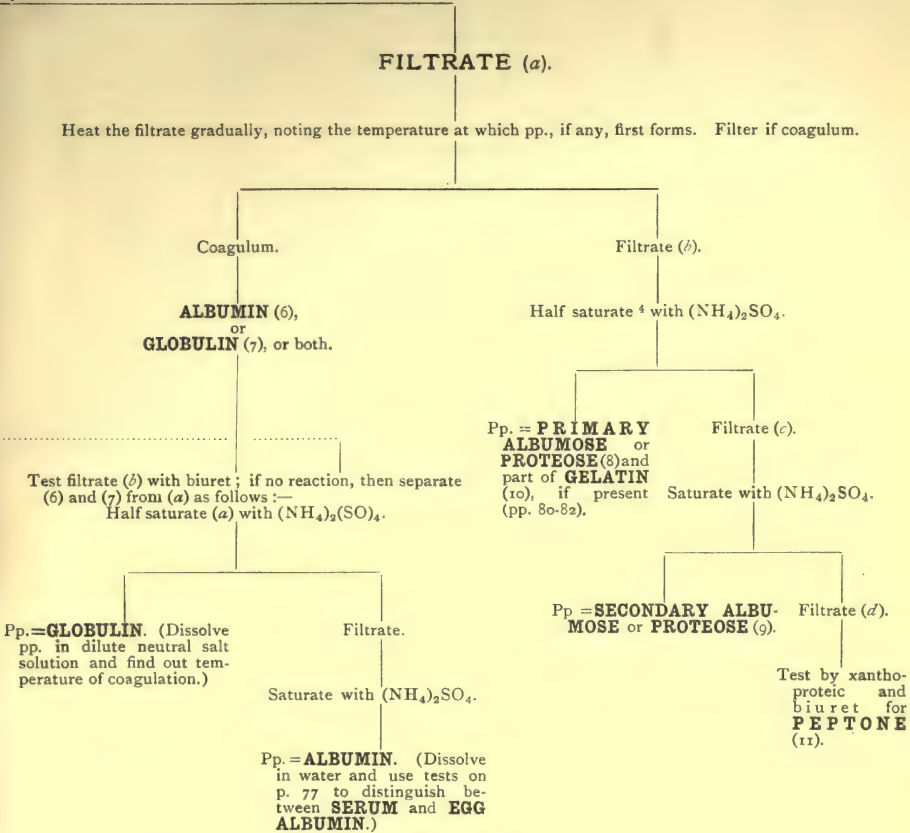
³ Prove that this pp. is protein in nature by colour tests (an alkaline solution of sodium urate yields a Test also the filtrate from each protein pp. by the chief colour reactions, to see if it be protein-free or not.

⁴ Care must be taken to test for polysaccharides, the precipitates obtained on saturation with salts.

NOTE.—O.S. = Original Solution. Pp. = Precipitate.

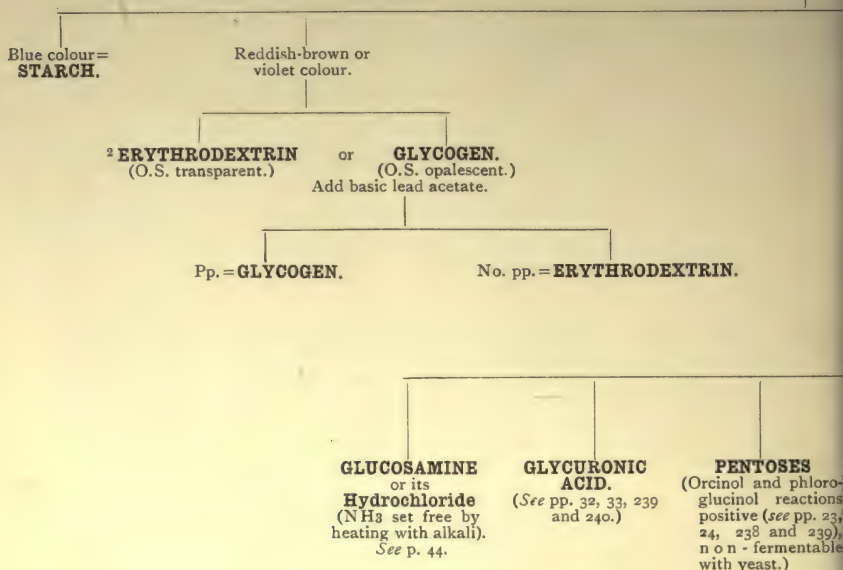
PROTEINS.¹

NEUTRALISE WITH VERY DILUTE CAUSTIC SODA IN THE FORMER IF A PP. FORM, FILTER IT OFF. A NEUTRAL SOLUTION OF A IN THE PRESENCE OF BILE SALTS² YIELDS A PRECIPITATE, WHEN



SCHEME III.—

RENDER THE SOLUTION NEUTRAL OR FAINTLY ACID. NOTE ANY
ADD A FEW DROPS OF A SOLUTION OF



¹ Before proceeding to test the aqueous extract of the solid or the O.S. (if the unknown substances be given in

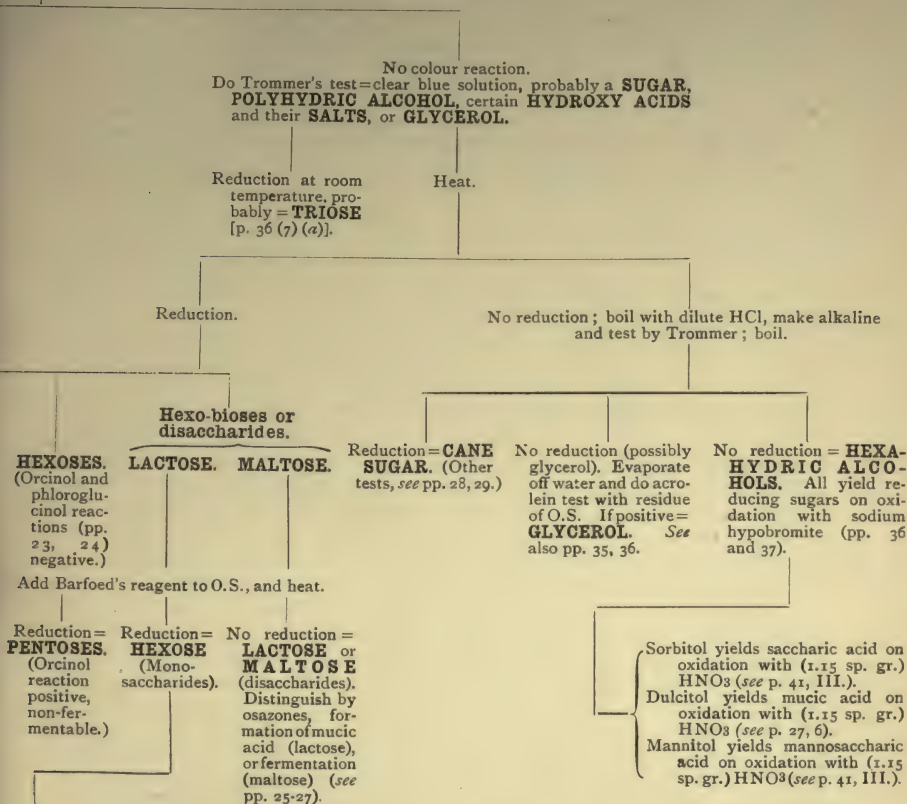
² When the solution contains starch and erythrodextrin, the two may be separated in the following way:—Add allow to stand for a few minutes, and filter through filter paper moistened with ammonium sulphate solution (2 vols. of is obtained. Compare with colour yielded by O.S. on addition of iodine. The starch has been precipitated and

³ The mannose solution requires to be boiled with the reagent for several hours before separation of the osazone is

CARBOHYDRATES.¹

DISTINCTIVE PHYSICAL CHARACTERS, SUCH AS OPALESCENCE.

IODINE IN POTASSIUM IODIDE.



	Fermentation.	Oxidation with (1.15) HNO ₃ .	Phenylhydrazine acetate in the cold.	Boiled with Phenylhydrazine acetate.	Resorcinol.	[α] _D .
Glucose	+	Saccharic acid	—	Glucosazone	—	+52°.5
Galactose	Very slow	Mucic acid	—	Galactosazone	—	+81°
Mannose	+	Mannosaccharic acid	Insoluble hydrazone	Glucosazone ³	—	+14°
Fructose	+	Two very soluble acids	—	Glucosazone	+	-93°

solution), it must be rendered protein-free in the usual way (boiling, slight acidification, with acetic acid, and filtration). 2 vols. of a saturated solution of ammonium sulphate (10 c.c.) to 1 vol. (5 c.c.) of the original solution, mix thoroughly, saturated solution and 1 vol. water). Add a drop or two of iodine solution to the filtrate—a red-brown or violet colour retained by the filter paper. Many samples of commercial dextrin contain a high percentage of soluble starch. complete; whereas glucose yields its osazone after being boiled for 15 to 30 minutes with the reagent.

Scheme IV.

Identification of the constituents of natural fluids of physiological importance.

I. Note the colour of the solution.

1. A light yellow aqueous solution may be:—(1) **blood serum**; (2) **blood plasma** (usually oxalate plasma); (3) a **gastric digest**; (4) a **pancreatic digest**; or (5) **normal urine**.

(1) *Frequently contains traces of oxyhæmoglobin due to partial hæmolysis. Examine with the spectroscope. Note the alkaline reaction of the solution. Prove the presence of coagulable protein, and test the filtrate from this coagulum for glucose and chlorides. Prove also that this filtrate is protein free (biuret reaction negative). Traces of acid metaprotein, produced during heat coagulation, may be present. If the blood serum is not quite fresh, glucose may be absent. Identify and separate the serum albumin and serum globulin present in the original solution.*

(2) *Examine in the same way as (1). Then half saturate a portion of the o.s. with NaCl. A protein precipitate indicates the presence of fibrinogen. Ascertain whether clotting occurs at 30° when calcium chloride solution and, if necessary, thrombin are added (see p. 159). If the presence of "salted" plasma be suspected, test as described on p. 158 II. (a).*

(3) *Determine reaction with litmus and congo-red papers. If the solution react acid to both, apply Günzburg's test. Test also for lactic acid (pp. 104 to 108). Detect and separate products of proteolysis, if present (pp. 113 to 115). If the fluid be a sample of the gastric contents, it may also contain products of amylolytic digestion. Therefore test with iodine for starch or erythrodextrin. Free a portion of the solution from coagulable protein by adding sodium acetate and heating, and test a portion of the filtrate for reducing sugar. If reduction occurs, concentrate the remainder of the filtrate to one-fourth its original bulk and add 9 times its volume of alcohol. Filter off the precipitate of proteoses and polysaccharides, dilute the alcoholic filtrate with a little water and drive off the greater part of the alcohol by heating the solution in a porcelain basin on the water bath. The sugar may then be identified in the way already described (Scheme III.).*

(4) *Note the reaction of the solution. Detect and separate, so far as possible, the products of tryptic digestion such as proteoses, peptone,*

tryptophane, and other amino-acids, especially leucine and tyrosine (pp. 121 to 124). Test also for possible products of amylolytic digestion such as erythrodextrin and maltose.

(5) Determine the reaction and specific gravity of the solution. Note the odour. Examine any sediment microscopically (see pp. 244 to 247). Identify by chemical tests the chief inorganic (e.g. chlorides, phosphates, sulphates, etc.) and organic constituents of urine (e.g. urea, creatinine, uric acid, etc.).

When a urine contains pathological constituents, or a pathological increase of some normal constituent (e.g. increased excretion of ethereal sulphates), the student need only determine the nature of the pathological constituent, or of the deviation from the normal composition of urine. In many cases, such problems can only be solved by a quantitative examination (see especially pp. 226 to 240).

2. A greenish-brown solution may contain bile pigment. Apply Gmelin's test. If this test be positive, examine the solution for the other constituents of bile (pp. 146 to 148).

3. A red solution may contain oxyhæmoglobin or some other derivative of blood pigment.

Determine the reaction of the solution, and identify the pigment by spectroscopic, and, if necessary, by chemical examination. If in doubt refer to plates of absorption spectra. If the solution be neutral, it may contain HbO_2 or HbCO . If distinctly alkaline, it may contain reduced alkali hæmatin. If strongly acid, it may contain acid hæmatoporphyrin. More rarely, the solution may contain metallic hæmatoporphyrin either in alkaline or in an organic acid solution, or alkaline hæmatoporphyrin (a brownish-red alkaline solution showing 4 absorption bands). A somewhat violet-red solution may contain reduced hæmoglobin. Further, a red solution may contain alkaline methæmoglobin.

(4) A brown solution of neutral reaction may contain neutral methæmoglobin. If acid, the brown solution may contain acid oxyhæmatin, or, if alkaline, it may contain alkali oxyhæmatin. Fairly concentrated solutions of urobilin are also brown, and may be neutral, acid, or alkaline in reaction (see plate of absorption spectra). Refer to pp. 164 to 182 for details regarding methods of identification.

II. A white opaque fluid may be an emulsion such as milk.

Examine microscopically for highly refractive fat globules. Determine the reaction and specific gravity of the fluid. Shake up with ether. Many artificial emulsions become clear when the fat is extracted by ether. Milk remains opaque after extraction with ether, but clears when dilute caustic alkali is added. Separate the ethereal solution, and evaporate the ether on the water bath. Note the oily character of the residue, and test for neutral fat by the methods given on pp. 57 to 59. Render a portion of the opalescent aqueous layer faintly

SCHEME IV.

acid with acetic acid, filter off the protein precipitate, and heat the filtrate to the boiling point. Filter off the coagulated protein, and test the filtrate for reducing sugar. If all the above chemical tests give positive results the fluid is almost certainly milk, and this conclusion may be verified by examining the original fluid in the way described on pp. 189 to 191.

Scheme V.

Identification of substances of physiological interest (other than proteins or carbohydrates), which may be present in an aqueous solution.

If the previous investigation has yielded negative results (i.e. if no positive reaction has been obtained on the addition of CuSO_4 and NaOH either before or after heating, and no colour change with iodine) proceed in the following way:—

I. Add drop by drop impure nitric acid (containing nitrous acid). One or other of the following results may be obtained:—

1. Bubbles of gas are evolved.—*Since the evolution of gas may be slow, it may readily escape observation. If in doubt, add an alkaline solution of sodium hypobromite to the o.s. If no gas be evolved the substances referred to under the heading I. are absent.*

*(1) The gas evolved on addition of impure nitric acid contains CO_2 , giving a precipitate with baryta water.¹ O.s. probably contains **urea or ammonium carbonate** (or guanidine carbonate), and the gas evolved is a mixture of N_2 and CO_2 . Confirm urea by noting that the reaction of the o.s. is neutral, and by concentrating a few drops of the o.s. on two microscopic slides. Cool the concentrated solution, and add a drop of pure nitric acid to one, and of saturated oxalic acid solution to the other. Examine the crystalline form of any crystals which may separate. If urease be available, it may also be used for detecting urea in the o.s. (see pp. 205 to 208 for other tests). If ammonium carbonate be present the o.s. will be alkaline in reaction, will yield volatile alkali on heating, and CO_2 on the addition of any acid.*

*(2) The gas gives no precipitate with baryta solution since N_2 only is evolved. O.s. may contain (a) an **amino-acid**, (b) an **amide** other than urea, e.g. **asparagine**, or certain **ureides**, e.g. **allantoin**, or certain **guanidine derivatives**, e.g. **creatine**, or (c) an **ammonium salt** other than the carbonate.*

Render the o.s. strongly alkaline by the addition of about twice its volume of 20 per cent. NaOH . Ammonia is rapidly evolved. O.s. contains an ammonium salt. Confirm by adding dilute caustic soda,

¹ For this test, a solution of nitrous acid, prepared by adding acetic acid to $\text{N}/1$ sodium nitrite, is preferable to impure nitric acid.

SCHEME V.

or solid magnesia, to the o.s. and heating gently. If an ammonium salt be present, NH_3 will be set free by the dilute alkali.

If ammonia is only gradually evolved when the alkaline solution is boiled vigorously, o.s. may contain one of the substances given under the heading (b). The complete identification of the substance may be impossible without prolonged investigation.

If no ammonia is evolved when the alkaline solution is boiled vigorously, an amino-acid may be present. Note, however, that certain amino-acids, e.g. arginine, yield NH_3 under these conditions. Remember also that ammonia may be present in combination with an organic acid, and therefore when ammonia is set free by the addition of a weak alkali such as MgO , further investigation is necessary.

When time is limited, the identification of the amino-acid may prove difficult or impossible. Further methods of identification will be given later for tyrosine and cystine.

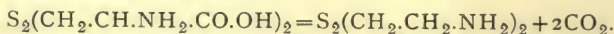
*The following tests may be applied. Concentrate a few drops of the o.s. on a microscopic slide to at least one-fifth of its original volume, allow to cool, and examine microscopically. Well-marked needles may consist of **glycine**. Plate-like crystals or spherules may be pure or impure **leucine**. Very fine needles may consist of **tyrosine**. Hexagonal plates may be **cystine**.*

*Add a little bromine water to the o.s. A violet colour indicates **tryptophane**.*

*Heat 2 or 3 c.c. of the o.s. with excess of cupric carbonate, and filter while hot. Concentrate a few drops of the filtrate on a slide or in a watch-glass, and allow to cool. If distinctly blue rhombic needles are seen on microscopic examination the original solution probably contains **glycine** or **alanine**; whereas, if the crystals of the copper salt are almost colourless, having only a very faint blue colour, an amino-acid of higher molecular weight such as **leucine** is present.*

If time permits, separate the amino-acid by concentrating the original solution, and allowing the acid to separate on cooling. Filter off the amino-acid, and dry in vacuo.

Heat a small portion in a dry test-tube. See p. 140 for the effect of heat on a number of amino-acids. When cystine is heated in a dry test-tube, it chars, yielding a yellowish-white vapour, which condenses as oily drops on the walls of the test-tube. The vapour is alkaline to litmus paper, and has an extremely pungent, somewhat garlic-like odour. Cystine yields amongst other products a base amino-ethyl disulphide, thus—



Dissolve a weighed quantity of the dry amino-acid in a known volume of water. If the solution be neutral to litmus, it is probably a monamino-monocarboxylic acid, if acid to litmus a monamino-dicarboxylic acid, and if alkaline, a diamino-acid. Salts of the two latter classes of amino-acids with bases or acids respectively may be

neutral to litmus. Further, salts of the dicarboxylic acids with strong mineral acids, e.g. glutamic acid hydrochloride react acid to congo-red. Free dicarboxylic monamino-acids contain one uncombined carboxyl group, and therefore react as monobasic acids on titration with N/10 NaOH, when sensitive litmus paper or phenolphthalein are used as indicators.

If the solution be neutral to or has been rendered neutral to sensitive litmus paper, its amino-nitrogen content can be determined by Sørensen's formaldehyde titration method. From the result obtained, the molecular weight of the amino-acid can frequently be calculated in the following way:—Let w grm. be the weight of amino-acid taken for analysis, and x be the number of c.c. of N/5 alkali required for neutralisation to phenolphthalein after the addition of neutral formaldehyde to the neutral amino-acid solution. Then, if the acid be a mono-amino mono-carboxylic one, its molecular weight (M)

$$= \frac{5000w}{x}.$$

If the acid be a dicarboxylic one, a preliminary acidimetric titration will be required in order to reach a definite conclusion.

Van Slyke's method may also be used in identifying the amino-acid present (see Chapters X. and XVIII.). Further, a determination of the percentage of nitrogen is frequently essential.

The results of such analyses may be confirmed by the preparation and analysis of the silver or copper salts of the unknown amino-acid (see p. 141), and by other methods given in larger text-books, or in the literature of the subject.

2. A precipitate separates. O.s. alkaline or neutral. The precipitate may be one of the following substances:—(1) **Urea nitrate** (o.s. neutral); (2) **uric acid** (o.s. Na, K, or Li salt); (3) **tyrosine** (o.s. NH_4 salt); (4) **cystine** (o.s. NH_4 salt); (5) **fatty acid** (o.s. Na or K soap); (6) **hippuric acid** (o.s., a salt); (7) **benzoic acid** (o.s. salt); (8) **salicylic acid** (o.s. salt); (9) **salicyluric acid** (o.s. salt); (10) **glycocholic acid** (o.s. Na salt); (11) **nucleic acid** (o.s. Na salt); (12) **skatole**, or rarer substances such as **indole acetic acid** (gives red nitroso-indole reaction with nitrous acid), and a number of aromatic acids such as **phenylacetic**, etc.

(1) *Identify urea by methods already described.*

(2) *Heat the solution after the addition of a few drops of nitric acid. The precipitate dissolves and gas is evolved. Apply the alloxan test, which will be positive if a urate be present in the o.s. A urate, if present, should have been detected at an earlier stage of the investigation (see p. 415).*

(3) *The precipitate dissolves readily in slight excess of the acid. Boil after the addition of nitric acid. The solution becomes yellow. Cool and add ammonia. The colour deepens to orange; (8) and (9) and a large number of other monohydric derivatives of benzene*

SCHEME V.

give a similar colour reaction. Add FeCl_3 to o.s. No colour change occurs. No precipitate is given on adding Br_2 dissolved in water to o.s. Heat o.s. with Millon's reagent. A deep red coloured solution, from which a red precipitate gradually separates, is obtained. Acidify a small portion of the o.s. with acetic acid and concentrate a few drops on a microscopic slide. Fine needle-shaped crystals separate out on cooling.

(4) Precipitate dissolves in slight excess of nitric, or other mineral acids such as HCl or dilute sulphuric. Acidify a portion of the o.s. with acetic acid, concentrate a few drops of the suspension or solution by evaporation on a microscopic slide, allow to cool, and examine microscopically (hexagonal plates). Test for unoxidised sulphur by boiling o.s. with a little caustic soda solution, and adding a drop of dilute lead acetate solution or of sodium nitroprusside solution.

Place about 0.02 gm. of the substance in a test-tube, add about 3 c.c. $\text{N}/10\text{HCl}$, and heat to the boiling point until the substance has dissolved. Then add a small quantity of stannous chloride (about 0.1 gm.), and boil for about 2 minutes. Cool slightly, add excess of finely powdered calcium carbonate, and shake up until effervescence of CO_2 has ceased. Filter, and add a few drops of dilute ferric chloride solution to the filtrate. A transitory blue colour appears, if cystine were present in the o.s. Cystine is reduced by stannous chloride to cysteine, and the latter forms a dark blue ferric salt. The blue solution is rapidly decolorised, because ferric chloride oxidises the cysteine to cystine, and the latter amino-acid does not form a coloured ferric salt.

Dissolve a small quantity of the substance (0.01 gm. or less) in a few drops of dilute ammonia (1 in 4), add about 2 c.c. of water, and a small quantity of zinc dust, boil for a minute or two, and filter. Add a few drops of 5 per cent. sodium nitroprusside solution to the filtrate, and a little solid ammonium sulphate. A deep red colour develops. On standing, the colour changes to a brown-red and gradually fades. The above test is negative with cystine; but positive with cysteine, into which the former is converted by the reducing action of zinc dust.

(5) Acidify o.s. with dilute sulphuric acid and heat. The precipitate melts to form an oil which rises to the surface. If the vapour have an acid reaction, and rancid odour, volatile fatty acids are also present.

Hold the warm test-tube very obliquely under the tap, and rotate the tube continuously until the mixture of fatty acids has solidified on the cooled sides of the test-tube. Pour off the dilute sulphuric acid into another test-tube, replace it with water, heat until the fatty acids melt, shake up thoroughly, and cool in the way already described. By repeating the washing, the mixture of fatty acids can be completely freed from sulphuric acid and glycerol. The above method is only satisfactory when the percentage of oleic acid or other unsaturated fatty acids in the mixture is low.

Remove a small portion of the solid fatty acids from the test-tube, dry between filter papers, and heat with large excess of finely-

powdered anhydrous acid potassium sulphate in a hard glass test-tube. No odour of acrolein can be detected.

If time permits, determine the melting point, or, preferably, the temperature of solidification of the mixture of fatty acids.

If a sufficient quantity of the fixed fatty acids has been separated, completely dry at least 0.5 gm. in vacuo. Weigh accurately about 0.5 gm. and dissolve in pure neutral ethyl-alcohol containing about one-fifth its volume of pure neutral ether, and titrate with N/10 alcoholic or aqueous KOH, using phenolphthalein as indicator.

Let w be the weight of fixed fatty acids taken, and x the number of c.c. N/10 alkali required for neutralisation. Since the acids are monobasic, their mean molecular weight is therefore equal to $\frac{10,000w}{x}$. When

several grammes of the mixture of fatty acids are available, the mean molecular weight may be more accurately determined by titrating about 3 grms. first with N/2 KOH, and completing the titration with N/10 KOH.

If glycerol be present in the o.s., it passes into the acid aqueous layer, and may be isolated by the method mentioned on p. 34. The separation is somewhat tedious.

Note.—When dilute sulphuric acid is added to the o.s., no precipitate may separate; but an acrid acid vapour may be evolved. The odour is intensified by heat. If no oil separates from the warm solution, the volatile fatty acids present probably have a lower molecular weight than $C_5H_{10}O_2$ (solubility of valerianic acid 1 in 30).

Acidify a portion of the o.s. with dilute HCl and saturate with calcium chloride. Any oil which separates may contain **butyric or propionic acid**. If no oil separates, the volatile fatty acids present may be **acetic or formic**. Add silver nitrate to the original neutral solution. If a white precipitate separates, and if on heating the precipitate becomes greyish-black, owing to the separation of silver, formic acid is probably present. No such reduction to metallic silver occurs if acetic alone be present. Formates also reduce $HgCl_2$ in a similar way. Both formates and acetates give a red colour with $FeCl_3$ solution. For fuller details the reader is referred to works on organic analysis.

(6) Acidulate the o.s. with dilute sulphuric acid, and shake thoroughly. A crystalline precipitate separates. Examine a portion microscopically for characteristic crystals (see Plate I.). Filter off a portion of the crystals, dry, and prove that they contain nitrogen. Heat another dry portion in a dry test-tube (see p. 220). Shake up one portion of the suspended crystals with ether and another portion with ethyl acetate. They dissolve in the latter solvent but not in the former.

Add drop by drop dilute $FeCl_3$ solution to the original neutral solution; a yellowish-orange precipitate separates.

(7) Acidify o.s. with dilute sulphuric acid. A crystalline pre-

SCHEME V.

precipitate separates. Shake up a portion of the suspended crystals with ether. They dissolve. Filter off a portion of the crystals, dry, and heat one portion in a dry test-tube. The substance melts, sublimes, and yields a vapour having a pungent aromatic odour. The substance is free from nitrogen. Add dilute FeCl_3 to original neutral solution. A yellowish-orange precipitate separates.

(8) On acidifying with dilute mineral acid, a crystalline precipitate, which is soluble in ether and chloroform, separates. Heat some of the crystals in a dry test-tube. The substance melts and yields a vapour having the odour of phenol. The latter reaction is more marked if the substance be heated with excess of soda lime. Add a few drops of dilute FeCl_3 to o.s. The solution acquires a violet colour, which is discharged by the addition of dilute mineral acids but not by acetic acid. The substance does not contain nitrogen.

(9) Acidify o.s. with dilute H_2SO_4 . A crystalline precipitate, soluble in ethyl-acetate but insoluble in ether, separates. Precipitate contains nitrogen. The o.s. gives a deep violet colour with FeCl_3 solution.

The o.s. (8) and (9) do not yield precipitates on acidulation with acetic acid; while (7) rapidly yields a precipitate, and (6) is slowly precipitated.

(10) Acidify o.s. with dilute sulphuric acid. A precipitate usually separates, although glycocholic acid may remain in solution if much taurocholic acid be also present. Add ether and shake. The precipitate remains undissolved, and may even be increased in amount. O.s. has a distinct bitter taste, and gives surface tension test with sulphur, and Pettenkofer's reaction. O.s. yields no precipitate with acetic acid.

(11) Add dilute H_2SO_4 to o.s. An amorphous precipitate (which readily dissolves on heating) separates. The precipitate when filtered off and dried will be found to contain N and P. Add acetic acid to o.s. No precipitate separates. For additional tests, see pp. 130, 131.

(12) See pages 130, 131, and 222.

3. A colour change occurs when impure nitric acid (i.e. containing nitrous acid) is added to the solution.

(1) A yellow colour, which is intensified by heating, develops. Cool and add NH_3 . The colour changes to orange. This reaction indicates phenol, or a phenol derivative such as tyrosine, saligenin, salicylic acid, or other hydroxy-aromatic acids (see pp. 221 and 222). All give a red colour when heated with Millon's reagent. Phenol and salicylic acid give precipitates with excess of bromine water; whereas tyrosine does not yield a precipitate. Phenol gives a bluish-violet colour with FeCl_3 solution, salicylic acid a violet colour, saligenin a bluish-violet, and tyrosine no colour change.

(2) A red colour develops. Shake up the red solution with a little amyl-alcohol. The red colouring matter is extracted by the alcohol. The o.s. contains indole or some indole derivative, such as indole acetic acid. Certain indole derivatives such as tryptophane do not

yield the red colouring matter, but give Hopkins and Cole's reaction with glyoxylic acid.

(3) **A violet or bluish-violet colour develops.** The solution may contain indican. Apply Jaffe's test to the o.s. (pp. 218 and 219).

(4) **A series of colours develops.** Bile pigment (Gmelin's test) may be present. Blood pigment, especially when present as hæmatin, gives a green colour more rapidly on heating than in the cold.

II. Add a few drops of 5 per cent. sodium nitroprusside solution to the o.s.

(1) **A violet colour indicates a soluble sulphide.** Confirm by lead test, and by the evolution of H_2S after acidifying and heating the o.s.

(2) **Add sodium nitroprusside and NaOH solutions.**

(a) **A red colour discharged by acetic acid indicates creatinine.**

(b) **A red colour deepened to reddish-violet by the addition of acetic acid indicates acetone or aceto-acetic acid.**

(c) **A reddish-violet colour changed to blue by acidification with acetic acid indicates indole.**

III. Add dilute $FeCl_3$ solution to the original neutral solution.

If the latter be acid, neutralise by adding a little ammonia and boiling off excess of alkali. If alkaline, add a little NH_4Cl , and boil off excess of ammonia. Then cool and add $FeCl_3$ solution.

(1) **A bluish-violet colour probably indicates phenol or saligenin.** $FeCl_3$ yields coloured compounds with a large number of monohydric and polyhydric benzene derivatives. The reader is referred to works on organic analysis for a more complete description of these reactions.

(2) **A violet colour not discharged by acetic probably indicates a salicylate.**

(3) **A dark red or reddish-violet colour indicates aceto-acetic acid or its ethyl ester.** Phloridzin, antipyrine, thiocyanates, and salicylates give somewhat similar colour reactions (see p. 241). The red colour given by all these substances, with the exception of thiocyanates, is discharged by HCl.

The following slight modification of Hurtley's test may be used in the identification of aceto-acetic acid or its ethyl ester. The following description gives the result of the reaction with the ester. This form of the test does not appear to be so delicate as Hurtley's original method, but has certain advantages for class work.

Place 5 c.c. of a dilute aqueous solution of ethyl-aceto-acetate in a test-tube, add 5 or 6 drops of N/1 sodium nitrite, and 7 or 8 drops of N/1 HCl. Mix thoroughly and allow the solution to stand for 10 to 20 minutes, preferably for the longer period. Add excess of magnesium oxide and shake thoroughly. If much aceto-acetic acid

SCHEME V.

be present, the solution acquires a bright yellow colour owing to the formation of a magnesium salt of isonitroso-acetone. The latter substance yields colourless solutions; whereas its salts with Mg, Ca, Na, K, and NH_4 are yellow. Filter off the excess of magnesium oxide, and add a few drops of a freshly prepared aqueous solution of a ferrous salt to the filtrate. The solution acquires an intense dark blue colour (see p. 242, 4). This test is negative with acetone.

(4) **A fainter red colour** may indicate the presence of a **formate, acetate, or of glycine.**

(5) **A lemon-yellow colour** indicates **lactic acid** or other **hydroxy-aliphatic acid.** The test should be repeated with an aqueous solution of the residue from an ethereal extract (see p. 108, 7).

(6) **A bright green colour** indicates **adrenaline** or other **catechol derivative.** Confirm by adding a few drops of iodine solution ($\text{N}/100$), shaking up, and removing excess of iodine by again shaking up with ether. A rose-red colour in the aqueous layer indicates adrenaline. For other tests, e.g. persulphate and mercuric chloride tests, see pp. 90-92 of "*The Simpler Natural Bases*," by G. Barger.

Adrenaline also reduces Folin's "uric acid reagent" [pp. 211 and 212, 6, (d) and (e)]. The reducing power of adrenaline on this reagent is three times as great as that of uric acid. A delicate colorimetric method has been based on this reaction (Folin, Cannon, and Denis).

(7) A distinct but transient **blue colour** indicates **cysteine.** Confirm by testing o.s. for unoxidised sulphur.

IV. Add a few drops of a saturated aqueous solution of bromine to o.s.

(1) *A violet colour indicates **tryptophane.** Extract the coloured solution by shaking with amyl alcohol. The colouring matter dissolves in the alcohol.*

(2) A white precipitate which becomes yellow on the further addition of bromine water indicates phenol or a phenol derivative, e.g. salicylic acid. Many dihydric derivatives of benzene yield similar precipitates.

V. Apply Molisch's test (p. 19). If this test be positive, other tests for carbohydrates negative, and biuret test also negative, a glucoside may be present.

Add 5 drops of concentrated hydrochloric acid to 5 c.c. of the o.s. and heat in the boiling water bath for about 30 minutes. Cool, neutralise, and apply Trommer's test. If reduction occurs, a glucoside is probably present. Note whether any odour, e.g. that of bitter almonds, is detectable. Also note whether any precipitate (e.g. phloretin) separates during the heating. Add FeCl_3 solution to o.s. A deep reddish-violet colour indicates phloridzin.

If the latter test be negative, and Trommer's test positive after acid hydrolysis, place a few c.c. of the o.s. in a test-tube, add a few drops of an emulsin solution, and a drop of toluene. Fix loosely in

the mouth of the test-tube by means of a cork, a strip of filter paper, which has been previously dipped in saturated picric acid solution, dried, dipped in 10 per cent. sodium carbonate solution, and again dried. Immerse the test-tube in water at 40° for half an hour. If HCN be evolved the paper will become orange-red, rose-red, and finally brick-red (E. F. Armstrong) owing to the formation of sodium isopurpurate. The solution has also a distinct odour of bitter almonds owing to the formation of benzaldehyde. If the above results are obtained, the glucoside is probably **amygdalin** (see pp. 46 and 47).

If the above tests are negative with the exception of a positive Trommer's test after acid or enzyme hydrolysis, add a few drops of ferric chloride solution to the glucoside solution which has been acted on by emulsin. A bluish-violet colour, due to the presence of saligenin, is obtained if the glucoside be **salicin**.

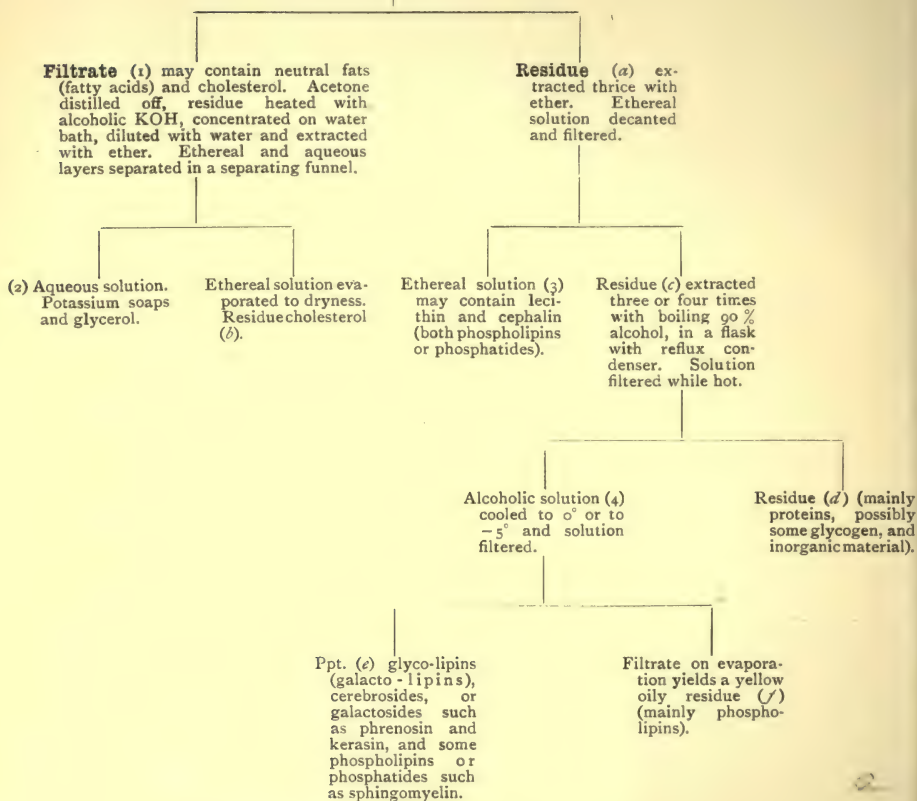
VI. If tests I. to V. give negative results, add silver nitrate solution as long as a precipitate, if any, continues to increase. Then add excess of ammonia. If the precipitate does not dissolve, silver compounds of the purine bases are present. The precipitate may be filtered off, decomposed with dilute HCl, the acid filtrate oxidised with Br₂ water, and the alloxan test applied [p. 213 (2)]. If the latter test be positive guanine, xanthine, or a xanthine derivative may be present. If negative, the purine base may be hypoxanthine or adenine.

The reaction of the o.s. will probably be distinctly acid to litmus, but the purine bases may also be dissolved in dilute aqueous solutions of NaOH or KOH.

SCHEME VI.

PARTIAL IDENTIFICATION AND SEPARATION OF SOME LIPINS AND FATS OR SUBSTANCES INSOLUBLE IN WATER AND AQUEOUS ALKALIES, BUT SOLUBLE IN ORGANIC SOLVENTS.

Dried tissue extracted three times with about
twice its weight of cold acetone. Solution
decanted and filtered.



SUPPLEMENTARY NOTES TO SCHEME VI.

Since most of the lipins have a highly complex chemical structure, and since their separation involves selective extraction with many organic solvents, their chemical investigation cannot be satisfactorily carried out in class work. The above scheme is only intended to give a rough indication of the chief methods of separation.

The tissue (usually nervous tissue, which is particularly rich in lipins) is spread out in as thin a layer as possible and dried in a vacuum oven over anhydrous calcium chloride at 40° to 45°.

(b) Cholesterol may be identified by Salkowski's and Liebermann's tests (see pp. 149 to 150 for other properties). The latter test is only slightly interfered with by the presence of neutral fats. Further, since nervous tissue does not contain neutral fats, the cholesterol obtained from it by direct extraction with cold acetone is nearly pure. Cholesterol may be purified by precipitation from ethereal solution by means of an alcoholic solution of digitonin; but this method is unsuitable for class-work owing to the cost of digitonin, and the difficulty of decomposing its compound with cholesterol into the constituents.

(3) The hydrolysis of lecithin (pp. 92 and 93) and cephalin, and the separation of the products of hydrolysis are beyond the scope of an elementary text-book. The presence of phosphorus in the residue from (3) may be demonstrated by Neumann's method (pp. 252 and 253).

(e) The presence of a carbohydrate radicle in (e) may be shown by suspending a portion of (e) in water, boiling, cooling, and applying Molisch's test (p. 19, l). Suspend another portion of (e) in cold concentrated sulphuric acid. The concentrated acid gradually acquires a somewhat violet colour. Dilute the coloured solution with glacial acetic acid, and examine with the spectroscope. An absorption band will be seen in the green region of the spectrum extending from about $\lambda 560$ to $\lambda 530$ (centre of band about $\lambda 545$). Another ill-defined band is also sometimes to be seen.

The presence of phosphorus in (e) may be demonstrated by Neumann's method. The complete hydrolysis of the substances present in (e) with dilute sulphuric acid, preferably in methyl-alcoholic solution, is a slow process, and therefore unsuitable for class-work. The presence of galactose amongst the products of hydrolysis may be demonstrated by means of the reactions given on p. 22, 3.

The presence of phosphorus in (f) may be proved by Neumann's method; (f) may also be tested for glycerol by the acrolein reaction.

Most of the lipins, *e.g.* lecithin, and the galacto lipins being anisotropic may be distinguished from neutral fats which are isotropic by examina-

SCHEME VI.

tion with the polarisation microscope. On heating the solid lipins until completely melted, they become isotropic, and therefore invisible when examined between crossed Nicols at a temperature known as "the clearing-point." On cooling, bright anisotropic needle-shaped crystals appear on the dark background. Cholesterol, its esters, and mixtures of cholesterol with neutral fats show similar changes on heating. These properties have rendered possible the recognition of lipins in tissues by microscopic examination.

The presence of unsaturated, fatty acid radicles in phospholipins such as lecithin, and in neutral fats containing olein can be demonstrated microscopically and macroscopically by the fact that they become black when brought into contact with a dilute solution of osmic acid. The osmic acid (OsO_4) is reduced to a black substance which is either a lower oxide of osmium or metallic osmium. For details regarding these and other microchemical methods, the reader is referred to works on histology.

ADDENDUM.

Separation of the chief constituents of animal tissues.— When the proteins, and along with them the nitrogenous and non-nitrogenous extractives, have been removed by the methods given on p. 193, the lipins may be extracted from the dried residue by the methods just described. The final residue consists of scleroproteins, usually mainly collagen. The collagen may be partially hydrolysed by boiling with water, and thus converted into gelatin (see p. 81, 1).

Scheme VII.

Examination of solutions of substances of physiological importance in organic solvents.

THE following description is mainly limited to substances which are insoluble in water, aqueous alkalies, and acids. Many organic substances, *e.g.* urea, are soluble both in water and many organic solvents such as ethyl alcohol.

A. An alcoholic solution may contain—(1) higher fatty acids (also soluble as soaps in aqueous alkalies); (2) neutral fats; (3) cholesterol; (4) galacto- or phospho-lipins; (5) bile salts (also soluble in water); or (6) creatinine (also soluble in water, p. 216).

(1) Place 5 c.c. of the alcoholic solution in a small flask, add a drop of 1 per cent. phenolphthalein solution, and determine the degree of acidity by titration with N/10 KOH. If only 0.1 to 0.2 c.c. of N/10 alkali are required to give a faint permanent red colour, the alcoholic solution may be regarded as free from fatty acids.

(2) Concentrate a portion of the alcoholic solution to dryness on the water bath, and examine residue by the methods given on pp. 57 to 59.

(3) Identify cholesterol by methods referred to in Scheme VI.

(4) Identify galacto- and phospho-lipins by the tests given in Scheme VI.

(5) Concentrate the alcoholic solution on the water bath, cool, and add excess of ether or acetone. Allow the precipitate to settle for an hour or two. Decant and filter the supernatant solution, and dissolve a small portion of the resinous precipitate in water. Test small portions of the aqueous solution for bile salts by the sulphur surface tension test, and by Pettenkofer's test. A small portion of the precipitate may also be tested for the presence of nitrogen and sulphur.

B. An acetone solution may contain neutral fats, fatty acids, or cholesterol, but neither phospho-lipins, galacto-lipins nor bile salts.

C. An ethereal solution may contain neutral fats, fatty acids, cholesterol or phospho-lipins such as lecithin.

D. A chloroform solution may contain neutral fats, fatty acids, cholesterol, phospho- or galacto-lipins, but not bile salts.

SCHEME VIII.—DETECTION OF FERMENTS OR

EXTRACT THE POWDER WITH NORMAL SALINE SOLUTION, CONTAINING
IN THAT FORM) FOR THE FOLLOWING EXPERIMENTS. TEST

STARCH in neutral or faintly acid solution. Test for dex-
trins and maltose. If positive=**dia-
static ferment**
(saliva or pancre-
atic juice).

FIBRIN(washed)in
.2 per cent. HCl.
Solution=probably
pepsin (see p. 110).

FIBRIN in .2-5 per
cent. Na_2CO_3 .
Solution=probably
trypsin or similar
ferment (see pp. 119,
120).

**MILK IN NEU-
TRAL SOLU-
TION.** Clotting=
probably **rennin**
(gastric juice), or
similar ferment
(pancreatic juice),
(see pp. 111, 112,
126).

ENZYMES IN A POWDER OR SOLUTION.

TOLUENE, AND USE THIS EXTRACT OR THE ORIGINAL SOLUTION (IF GIVEN EFFECT OF THE SOLUTION ON THE FOLLOWING (AT 35-40°).

- (a) CANE SUGAR.
(b) MALTOSE.
(c) LACTOSE IN NEUTRAL SOLUTION.

Test to see whether (a) is transformed into a laevo-rotatory mixture of reducing sugars. If so = **invertase**. If (b) be transformed into glucose = **maltase**. If (c) be transformed into glucose and galactose = **lactase** (see pp. 45 and 126-128). If fermentation of (a) and (b) (in Einhorn's tube) = **yeast** (in suspension).

FIBRINOGEN IN A SOLUTION FREE FROM OXALATES. Clotting = fibrin ferment (see p. 159).

AMYGDALIN or SALICIN. (If the glucoside be hydrolysed, emulsin is present.) (See pp. 45-48.)



PLATES

SOME CRYSTALS OF PHYSIOLOGICAL IMPORTANCE

FIG. 1.—MONACID CALCIUM PHOSPHATE— CaHPO_4 . "Stellar Phosphates."

FIG. 2.—(a) AMMONIUM MAGNESIUM PHOSPHATE, or Triple Phosphate.—
Typical "Knife-rest" or "Coffin-lid" crystals.

OTHER FORMS OF THE TRIPLE PHOSPHATE.—

(α) Imperfectly formed "Knife-rests."

(β) "Feathery Phosphates."

(b) ACID AMMONIUM URATE.—"Hedgehog" crystals. Spherules with or without Spines (high power).

FIG. 3.—OXALATE OF LIME.—

(a) "Envelope-shaped."

(b) "Dumb-bells."

FIG. 4.—URIC ACID.—Some common forms.—

(a) "Whetstone," "Dagger," and "Rosettes."

(b) "Barrels."

(c) "Lozenge," "Cube," etc.

FIG. 5.—HIPPURIC ACID.

FIG. 6.—(a) UREA.

(b) NITRATE OF UREA.

(c) OXALATE OF UREA.

FIG. 7.—(a) LEUCINE (impure).

(b) TYROSINE.

FIG. 8.—CYSTINE.

FIG. 9.—CHOLESTEROL.

FIG. 10.—(a) PHENYLGLUCOSAZONE (low power).

(b) PHENYLMALTOSAZONE (high power).

(c) PHENYLLACTOSAZONE (high power).

PLATE I.



Fig 1

Fig 2

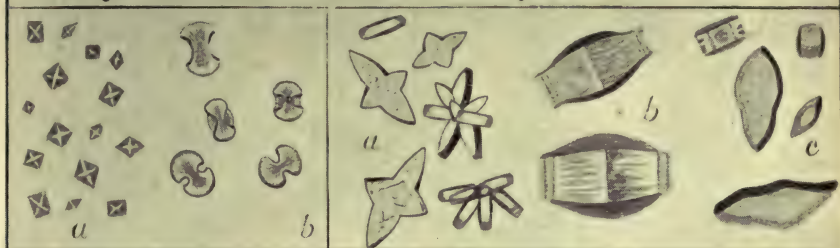


Fig 3

Fig 4

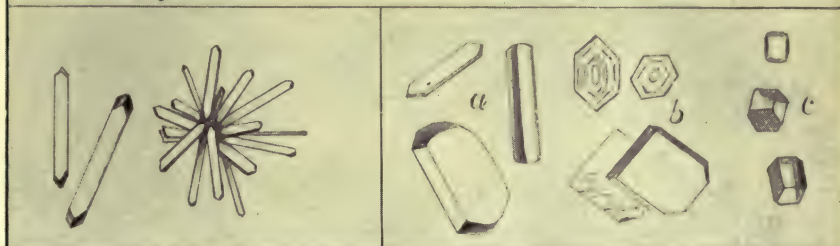


Fig 5

Fig 6

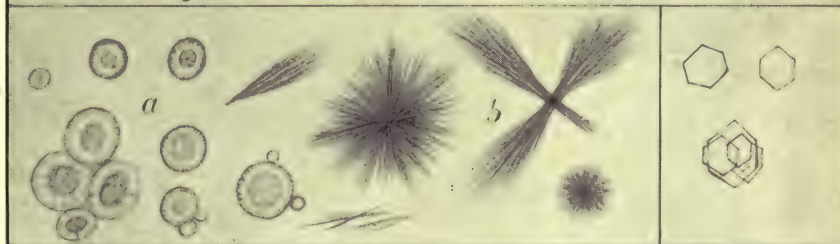


Fig 7

Fig 8

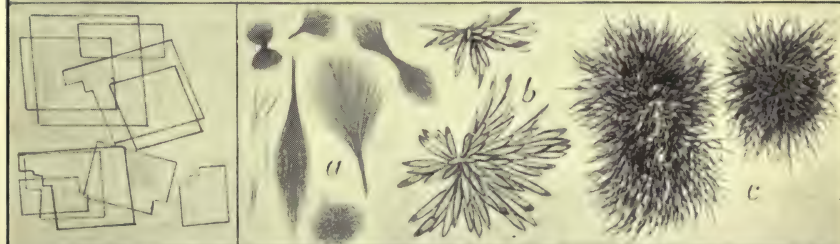


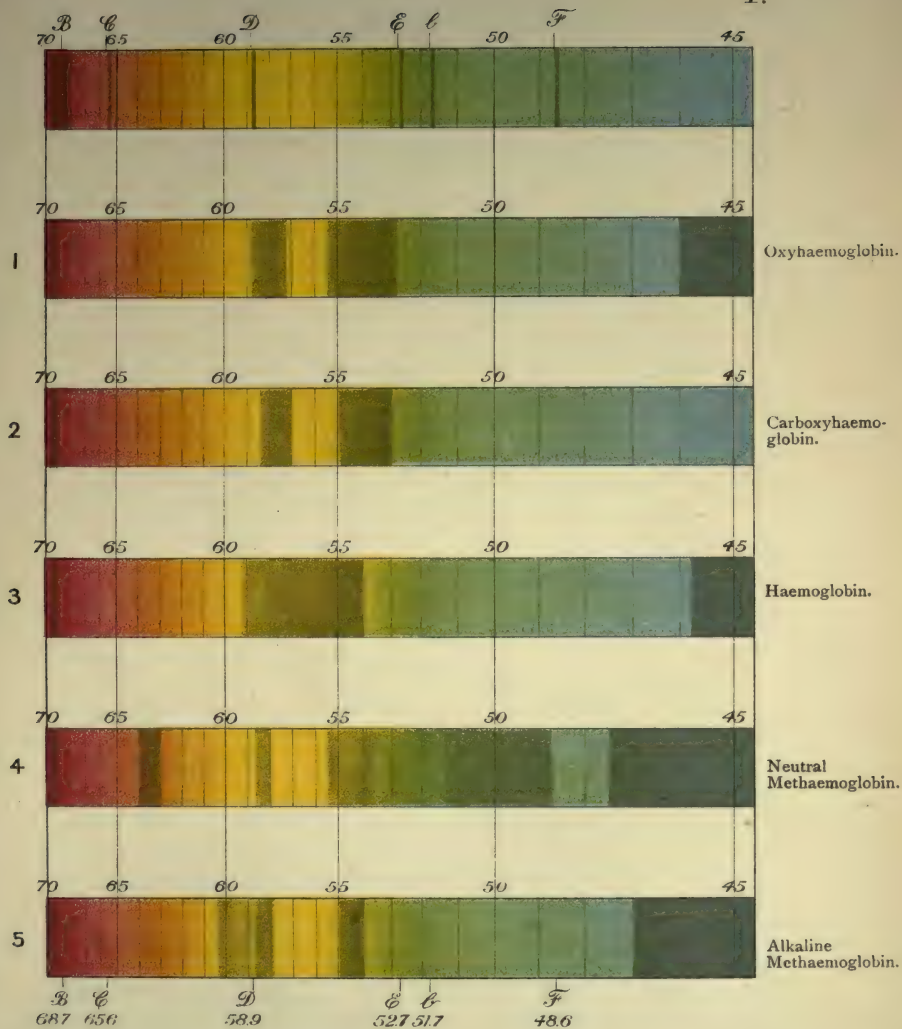
Fig 9

Fig 10

Re Hall.

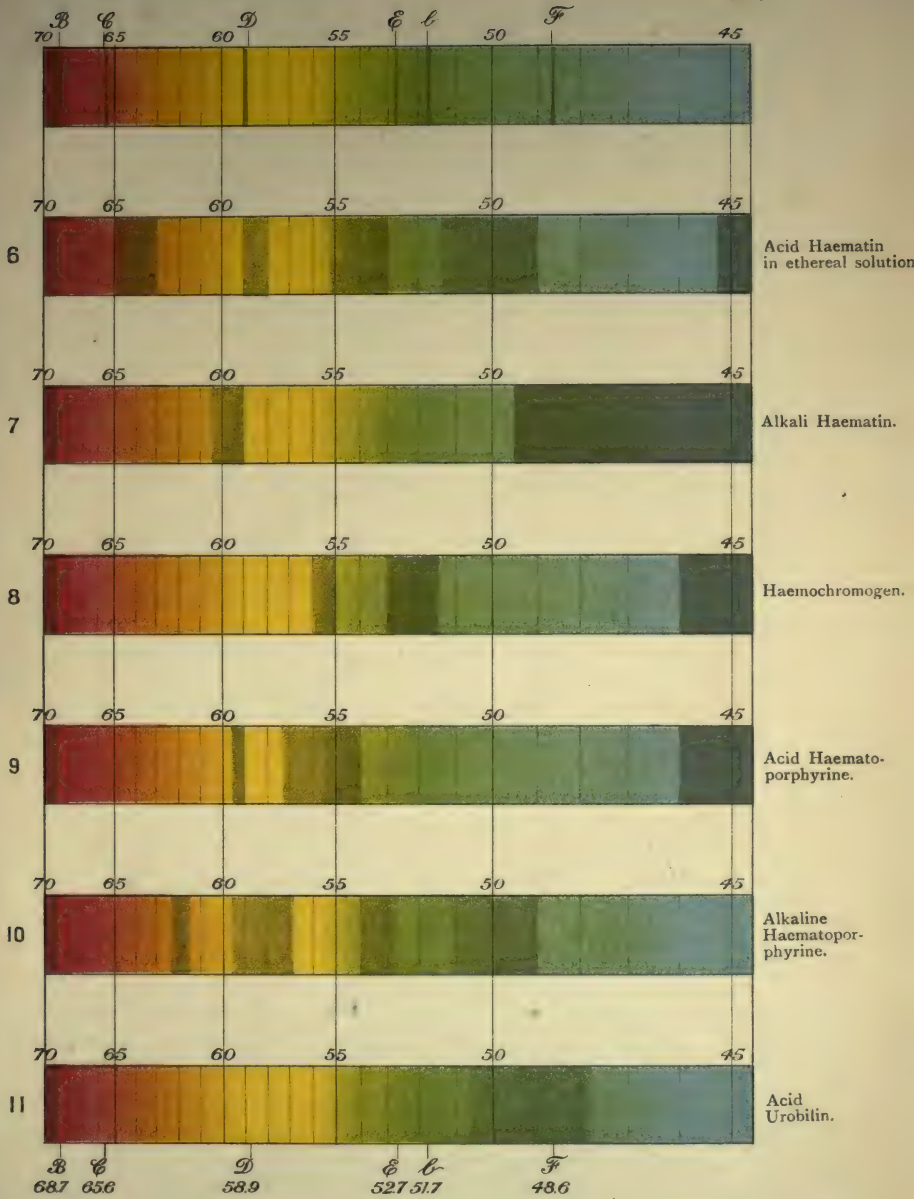


I.





II.





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